Effect of Cellular Positional Identity on Bone Regenerative Capacity for Tissue Engineering

Prajaneh, Saengsome

Awarding institution:
King's College London

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Saengsome Prajaneh

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Unit of Periodontology, Department of Restorative Dentistry
King's College London Dental Institute
King's College London

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ABSTRACT

The aim of this study was to investigate the stability of positional identity markers and phenotypic differences in isolated osteoblasts from distinct anatomic regions. In addition, the ability of heterotypic co-cultures to reprogramme site-specific *Hoxa* gene expression also tested.

Rat osteoblastic cells from femurs and calvariae were harvested as matched pairs of cultures from 4 male rats. Cells were expanded extensively in medium supplemented with FGF-2, and were shown to maintain their osteoblastic phenotype as characterised by alkaline phosphatase (ALP) staining, osteopontin (OPN), osteocalcin (OCN) expression and osteoblast-associated gene expression in long term culture.

Gene expression of cells was determined by quantitative RT-PCR. Differences in *Hoxa* gene expression as markers of positional identity were maintained for up to at least 10 passages, with calvarial cells remaining *Hoxa*-ve throughout. The transcription factors *Msx2* and *Irx5* were consistently more highly expressed in calvarial cells, whereas *Tbx3* expression was elevated in femoral cells. Expression of the osteoblast-associated genes *Bglap* and *Spp1* were elevated in femoral cells, and also associated with increased osteopontin secretion and bone nodule formation. *Runx2* was elevated in calvarial cells. Cells were also pre-labelled with fluorescent vital staining and co-cultured for 7 days prior to separating by fluorescence activated cell sorter to investigate the possibility of re-programming of *Hoxa* negative cells by direct contact with *Hoxa* +ve cells. However no evidence was seen of modulation of positional identity genes and phenotypes in these heterotypic cultures.

In conclusion, the results demonstrate persistence of expression of positional identity gene markers and phenotypic differences between femoral and calvarial osteoblasts for prolonged periods in culture. These data suggest that these differences in regionally defined osteoblasts are inherently programmed in the cells as a result of their embryological position. The results may have considerable implications when considering the transplantation of autologous cells in tissue engineering.

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CONTENTS

LIST OF FIGURES

Chapter 1:

Chapter 2:

Chapter 3:

LIST OF TABLES

LIST OF SUPPLIERS

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ABBREVIATIONS

$1,25(OH)_2D_3$	Vitamin D
5-HT	Serotonin
ABI	Applied Biosystems
AGRA	18-α-glycyrrhetinic acid
Alcam	Activated leukocyte cells adhesion molecule
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
Apo-E	Apolipoprotein E
ASP	Asialoprotein
ATF4	Activating Transcription Factor 4
ATP	Adenosine triphosphate
Atp5b/ATP5B	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide
b-ALP	Bone-specific alkaline phosphatase
β-ME	Beta-mercaptoethanol
B2m	β2 microglobulin
Bglap	Bone gamma-carboxyglutamate (gla) protein
BGP	Bone Gla protein
Bis-Tris	2-[Bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3propanediol
BLAST	Basic local alignment search tool
BMD	Bone Mineral Density
BMP/Bmp	Bone morphogenetic protein
Bmp/Cdmp	Bone morphogenetic protein /Cartilage-derived morphogenetic proteins
BMSC	Bone marrow stromal cell
BrdU	Bromo-deoxyuridine
BSA	Bovine serum albumin
BSPI	Bone sialoprotein I
BSPII	Bone sialoprotein II
c-Abl	Cytoplasmic protein-tyrosine kinase
Ca2+	Calcium ions

$Ca_{10}[PO_4]_6[OH]_2$	Hydroxyapatite
Cart	Cartilage paired-class homeoprotein
Cbfa1	Core-binding factor a1
cDNA	Complementary deoxyribonucleic acid
CM	Conditioned medium/media
CMFDA	5-Chloromethylfluorescein Diacetate
CMRA	CellTrcker^{TM} Orange
CMTPX	CellTrcker^{TM} Red ($C_{42}H_{40}ClN_3O_4$)
CO_2	Carbon dioxide
COL1	Collagen type 1
COL1A1	Collagen type 1 α1
Cq	Quantification cycle
Cre	cAMP response enhancer element
CS	Chondroitin sulphate
CSF	Colony-stimulating factor
°C	Degree Celsius
DAPI	4',6-diamidino-2-phenylindole
deltaFosB	Fos family proteins
dH_20	Distilled water
Dhh	Desert Hedgehog
Dkk/DKK	Dickkopf
Dll	Drosophila distal-less
Dlx	Distal-less homeobox
DMEM	Dulbecco's Modified Eagle's Medium
DMF	N,N-Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
ECM	Extracellular matrix
EDTA	Ethylene-diamine-tetra-acetic acid
EdU	5-ethynyl-2´-deoxyuridine
EGF	Epidermal growth factor
EIA	Enzyme Immunoassay

Eif4a2/EIF4A2	Eukaryotic translation initiation factor 4A isoform 2
ELISA	Enzyme linked immunosorbant assay
ER-α	Oestrogen receptor-α
ER-β	Oestrogen receptor-β
$ER^{-/-}$	Oestrogen receptor knock out
EtBR	Ethidium bromide
FACS	Fluorescence Activated Cell Sorters
FBS	Foetal Bovine Serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
Fn	Fibronectin
Fra-1	Serum-inducible protein
Fst	Follistatin
Fzd	Frizzled
g	G-force
G6PD	Glucose-6-phosphate dehydrogenase deficiency
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
Gdf	Growth differentiation factor
GFP	Green fluorescent protein
GJC	Gap-junctional communication
Gli3	zinc finger 3
GSK-3beta	Glycogen synthase kinase-3 beta
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HCl	Hydrochloric acid
hh	Hedgehog
Hox	Homeobox
IBMX	Isobutylmethylxanthine
Ibsp	Bone sialoprotein
IGF	Insulin-like growth factor
IgG	Immunoglobulin G
IMS	Industrial methylated spirits
Ihh	Indian Hedgehog
Irx	Iroquois related homeobox
I-Smads	Inhibitory Smads

KCl	Potassium chloride
KO	Knockout
KOH	Potassium hydroxide
Lmx1b	LIM homeobox transcription factor 1-beta
LRP/Lrp	Low-density lipoprotein receptor-related protein
Ltd.	Limited
µg	Microgram
µg/ml	Microgram per millilitre
µl	Microlitre
µm	Micrometre
M	Molar
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
MEM	Minimum Essential Media
Mesp2	Mesoderm posterior protein 2
MG-63	Human osteosarcoma cell line
$MgCl_2$	Magnesium chloride
mg	Milligram
mg/µl	Milligram per microlitre
mg/ml	Milligram per millilitre
mins	Minutes
ml	Millilitre
mM	Millimolar
M-MLV	Moloney Murine Leukemia Virus
MMP	Matrix metalloproteinase
MΩ/cm	Megohm per centimetre
mRNA	Messenger RNA
MSC	Mesenchymal stem cell
MSF	Migration stimulating factor
Msx	Mesh-less
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
mw	Molecular weight
NaCl	Sodium Chloride

$NaHCO_3$	Sodium bicarbonate
NaOAc	Sodium acetate
NaOH	Sodium hydroxide
Na_2CO_3	Disodium carbonate
Na_2HPO_4	Sodium phosphate
NBCS	Newborn calf serum
NCBI	National Center for Biotechnology Information
NCSCs	Neural crest-derived stem cells
NFQ	Non fluorescent quencher
ng	Nanogram
ng/ml	Nanograms per millilitre
NHS	National Health Service
nm	Nanometre
No-RT	No reverse transcriptase
NTC	No template control
NY	New York
OC/OCN	Osteocalcin
Osf2	Osteoblast specific factor 2
OP/OPN	Osteopontin
O.D.	Optical density
OPG	Osteoprotegerin
$OPN^{-/-}$	OPN knock out
Osx/OSX	Osterix
%	Percentage
p	Passage
Pax	Paired box
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	population doubling
PDGF	Platelet derived growth factor
PE	R-Phycoerythrin
PFA	Paraformaldehyde
PMS	Phenazine methosulphate
PNP	*p*-nitrophenol

PPARγ/PPARG	Peroxisome proliferator-activated receptor gamma
Prx	Peroxiredoxin
PTH	parathyroid hormone
PTH1R	type 1 parathyroid hormone receptor
QC	quality control
qRT-PCR	quantitative Reverse Transcription PCR
R26R	Cre-dependent lacZ reporter strain
rae28	Polycomb-group (PcG) gene Rae28
Raldh2	Retinaldehyde dehydrogenase 2
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
RPMI	Roswell Park Memorial Institute
RN18S1	RNA, 18S ribosomal 1
RNA	Ribonucleic acid
rpm	Revolutions per minute
rh	Recombinant human
rm	Recombinant mouse
RGD	Arg-Gly-Asp
ROR	Retinoic acid receptors
ROS	Rat osteosarcoma 17/2.8 cell line
R-Smads	Receptor activated Smads
RT	Room temperature
RUNX/Runx	runt-related transcription factor x
Sall1	Sal-like 1 (Drosophila) gene
SATB2	Special AT-rich sequence-binding protein 2
Sc1	Sclerostin
S.D.	Standard deviation
sec	Second
Sfrp	Secreted frizzled-related protein
Shh	Sonic Hedgehog
Shox	Short stature homeobox
SLRP	Small leucine-rich proteoglycan
Smad	Small mothers against decapentaplegic
Smoc	SPARC related modular calcium binding

SOST	Sclerostin
Sostdc1	Mice carrying mutations in *Wise*
SPARC	Secreted Protein Acid Rich in Cysteine
SPP	Secreted phosphoprotein
TAE	Tris-acetate-EDTA
Taz	Transcriptional coactivator with PDZ-binding motif
TEA	Triethanolamine
Tbx	T-box
Tg	Transgenic
TGF	Transforming growth factor
TMB	Tetramethylbenzidine substrate (3,3´ 5,5´ -tetramethylbenzidine)
tRNA	Transfer RNA
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
Twist	Twist-related protein
Ubc/UBC	Ubiquitin c
UK	United Kingdom
U	Unit
UTP	Uridine triphosphate
v	Version
V	Volts
WIF	Wnt inhibitory factor
Wnt1Cre::Z/EG	transgenic mice
Wnt7a	Wingless-related MMTV integration site 7A
Zic	Zinc finger protein
$ZnCl_2$	Zinc chloride

Chapter 1

Chapter 1: Literature review

1.1 Introduction

Tissue engineering is a rapid growing multidisciplinary research field which offers the opportunity to restore form and function of tissue concerning biology, medicine and engineering to improve health and quality of life (National Institute of Health, 2012). The novel therapeutic strategies have been developed according to cellular and molecular works in the past 2 decades. Tissue engineering involves three keys elements: scaffolds, signaling molecules, and cells which aims to induce tissues or organs to regenerate themselves, by providing mechanical, cellular, and molecular signals.

Therefore, focusing on bone and tissue engineering could be advantageous to improve clinical treatments such as bone grafting. Bone tissue engineering may be a ground breaking technology for reconstructive and regenerative treatments. Understanding the anatomy and processes of bone formation and regeneration not only at the cellular level, but also at the molecular level, may help to comprehend all the critical criteria. Mimicking the natural environment will allow cultured cells to behave in a more realistic way, and give a chance to study these biological systems in laboratory. The attempt to mimic nature may further develop bone grafting materials or bone grafting procedures to improve a predictable clinical outcome.

Bone is one of the most frequently considered options for transplantation (Giannoudis et al., 2005). Bone grafts have been used to replace, reconstruct or regenerate impaired bony defects for patients both in medical and dental fields. Autologous bone grafts are recognised as the gold standard for bone grafting procedures (Kneser et al., 2002). The success rate of implanted grafts which may incorporate to host skeletal sites through osteconduction, osteoinduction, osseointegration, osteogenesis processes are varied and depended on many circumstances such as recipient-site defects, donor-site morbidity, the need for vascularisation and the ability of osteogenic potential in grafted materials, local and systemic factors. Allograft, xenograft or alloplast materials have become an alternative option when an autograft is not available (Giannoudis et al., 2005; Jayakumar and Di Silvio, 2010). Up to date, the clinical success rate of bone grafting is still unpredictable.

Recently, evidence has shown that committed cells in adult mammals such as fibroblasts and mature osteoblasts do not have adequate diversity of their phenotypes when compared to embryonic stem cells. However, they may show considerable functional diversity according to their anatomical positions (Rinn et al., 2008a). For example, the human skin shows notable diversity in its structure and function across the body (Rinn et al., 2008a). Although all fibroblasts are morphologically similar, the comprehensive gene expression analysis of primary human fibroblasts from distinct anatomical sites has shown that gene expression patterns of cultured fibroblasts from different sites are remarkably distinct (Chang et al., 2002). Over many years, not only fibroblasts, but osteoblasts have also been studied. In fact, factors leading these signals to the diversity of osteoblast gene expression profiles remain unknown. Understanding the functional diversity between regionally committed cells may be needed to taken into consideration for selecting appropriate sources and methods when transplantation is required.

1.2 Bone formation

Bone is a highly organised, specialised form of connective tissue, metabolically active and constantly remodelled throughout life (Hughes et al., 2006). Bone is divided into compact bone and cancellous bone. There are two different ossification processes. Intramembranous ossification is formed by condensations of mesenchymal cells at the centre of ossification which transform into osteoblasts e.g. flat bones of the skull, parts of the clavicle, mandible, maxilla and periosteal surface of long bone. The other process is endochondral ossification which requires the formation and degradation of cartilaginous templates which occur in long bones (Ng et al., 1997).

Bone matrix is composed of approximately one-third of organic matrix and two-third of inorganic matrix by weight (Sodek and McKee, 2000). The main structural protein of the organic matrix is Type I collagen, which makes up approximately 90% of the matrix. It provides structure, strength, and elasticity of the mature bone and acts as a scaffold or template for initiation and preparing for later matrix mineralisation (Rossert and de Crombrugghe, 2002). The remaining 10% of the organic matrix consists of non-collagenous proteins such as osteopontin, osteocalcin, bone sialoprotein, osteonectin, and other components.

Osteopontin, also known as bone sialoprotein I (BSPI) or secreted phosphoprotein (SPP), is a glycoprotein (Oldberg et al., 1986). It is synthesised by a variety of cell types and is not only limited to bone cells. Along with bone sialoprotein, this appears to control nucleation and crystal growth during mineralisation including mediating attachment of bone cells to their matrix (McKee and Nanci, 1996). Its role in modulating osteoblast responses to mechanical loading is considered further in Chapter 6.

Osteocalcin or bone Gla protein (BGP) is a calcium binding protein. It makes up above 10% of the non-collagenous protein in the mature bone, but is present at much lower concentrations in embryonic bone. It is recognised to be the most bone-specific marker and its expression is restricted to mineralised tissue cells (Hughes and Aubin, 1998).

Bone sialoprotein, also referred to as bone sialoprotein II (BSPII), is not as extensively distributed in non-mineralised tissues as osteopontin. Both osteopontin and bone sialoprotein contain the Arg-Gly-Asp (RGD) peptide cell which have a crucial role during mineralisation in mediating attachment of both osteoblasts and osteoclasts to the bone matrix (Oldberg et al., 1986, 1988).

Osteonectin, or Secreted Protein Acid Rich in Cysteine (SPARC), was originally isolated from bone matrix (Termine et al., 1981) and serves as a nucleus for mineralisation and links collagen to hydroxyapatite which regulates the formation and growth of hydroxyapatite crystals. It is expressed in a wide range of both embryonic and adult tissues, cultured cells, including fibroblasts, endothelial cells, lung, ovaries and testes.

Other components of the organic matrix include the proteoglycans decorin and biglycan, which belong to the small leucine-rich proteoglycan (SLRP) family and consist of a protein core containing leucine repeats and chondroitin sulphate (CS) side chains. Their particular function is unknown. They can bind to transforming growth factor-β which may have an important role in their regulation. They can also bind to other matrix macromolecules, including collagen and appear to be involved in fibrillogenesis and mineralisation (Hoshi et al., 1999).

In addition, plasma-derived proteins such as albumin, small amounts of carbohydrate, and growth factors are also present in bone matrix, usually bound by other matrix molecules (Sodek and McKee, 2000). In general, bone matrix proteins appear to have an important role in the regulation of hydroxyapatite crystal deposition, including crystal orientation and size formation. Some of the components such as bone sialoprotein and osteopontin also mediate attachment of osteoblasts and osteoclasts to the bone matrix. In addition, growth factors are bound within the matrix and may have an important regulatory role concerning bone remodelling (Hughes et al., 2006).

The other two-thirds of bone matrix is an inorganic matrix consists of calcium and phosphate salts, mainly in the form of hydroxyapatite ($Ca_{10}[PO_4]_6[OH]_2$) crystals. The mineral forms thin, plate-like crystallites showing an ordered orientation within the collagen fibrils. It also has a major role in the bio-mechanical function of bone (Landis et al., 1996).

1.3 Bone development

In early development, the vertebrate skeleton is formed by cells from three distinct embryonic origins; cranial neural crest cells which form the craniofacial skeleton, paraxial mesoderm (somites) which forms the axial skeleton, and lateral plate mesodermal cells which form the limb skeleton (Olsen et al., 2000). In addition, bone formation is controlled by transcription factors, growth factors, cytokines, and extracellular matrix molecules (Figure 1-1) (Olsen et al., 2000).

Previous studies in mice and chick embryos have shown that crest cells from the caudal midbrain and rhombomeres 1 and 2 migrate into the first branchial arch, giving rise to maxilla, mandible, incus, malleus, and regions of the temporal bone, whereas cells in the second branchial arch give rise to stapes, styloid process of the temporal bone, and part of the hyoid bone, and are derived from rhombomere 4 (Olsen et al., 2000). Many genes encoding for transcription factors, such as *Dlx1*, *Dlx2*, *Dlx5*, *Msx1*, *Cart1*, *Hoxa1*, *Hoxa2*, *Hoxa3*, *Hoxb1*, *rae28*, *Twist, Pax*; and for cytokines and growth factors such as *BMP4, Fgf8, Tgf-α* and *endothelin-1* play important roles in craniofacial bone development (Olsen et al., 2000).

Most of the craniofacial bones are of neural crest origin (Olsen et al., 2000). However, studies in Wnt1-Cre mice using a conditional reporter gene, R26R, have demonstrated contributions of cells of distinct embryonic origins to the skull bones with neural crest-derived frontal bone and paraxial mesodermal-derived parietal bone (Chai et al., 2000; Jiang et al., 2002).

The somites give rise to the vertebrae and the dorsolateral portion of the ribs, the dermis of the dorsal skin, and the skeletal muscle of the body wall and the limbs. Somitogenesis occurs bilaterally, in a precisely timed rostro-caudal sequence. The *Notch-Delta* pathway and genes such as *Mesp2*, *Notch1*, *Dll*, *lunatic fringe Dll3*, sonic hedgehog (Shh)*, Pax1, Pax9*, and *Hox* play important roles in somitogenesis (Olsen et al., 2000).

The limb bones are produced by cells derived from the lateral plate mesoderm. The proximodistal growth patterning is directed by fibroblast growth factor signals from the specialised epithelial cells; anteroposterior patterning is directed by sonic hedgehog and dorsoventral patterning depends on secretion of Wnt7a (Olsen et al., 2000). *Gli3*, *Sall1*, *Hoxd13*, *Hoxa13*, *Bmp*/*Cdmp*, *Gdf* and *noggin* are genes controlled by sonic hedgehog and genes related to BMP signalling; *Fgfs* are genes expressed by the apical ectodermal ridge; *Tbx5* and *Tbx4* are transcription factor genes associated with establishment of limb identity; *Wnt7a*, *radical fringe*, *engrailed* and *Lmx1b* are genes associated with specification of dorsal-ventral properties (Figure 1-2) (Olsen et al., 2000).

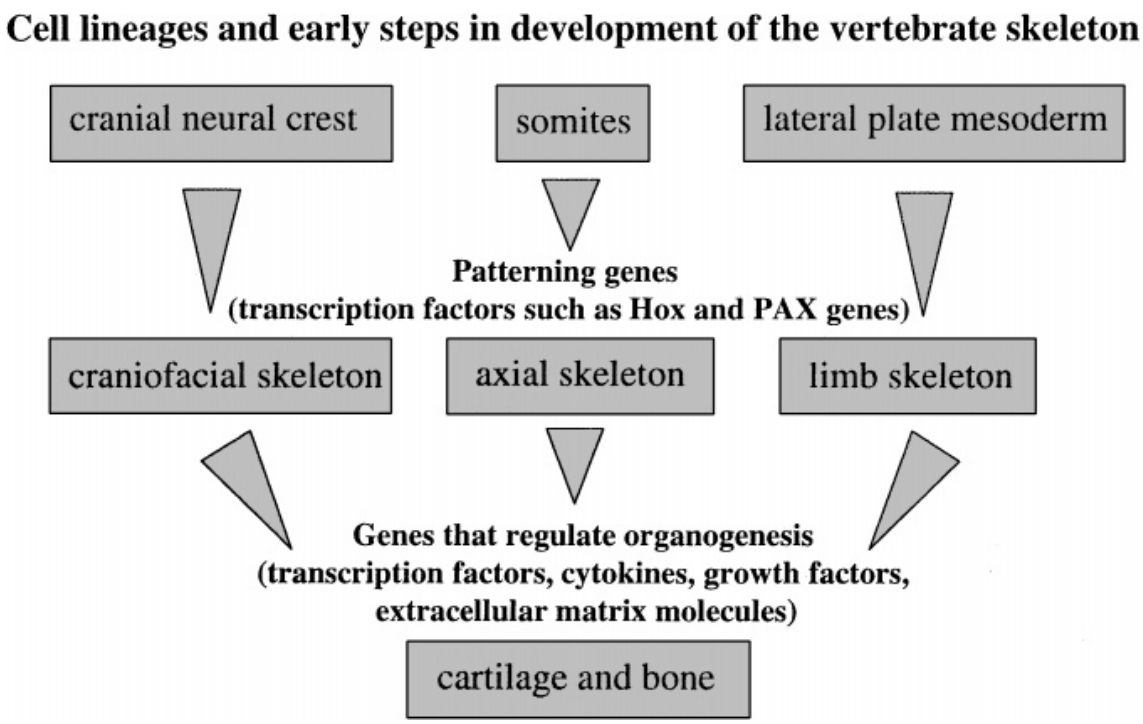


Figure 1-1: Diagram shows the formation of cartilage and bone controlled by transcription factors, cytokines, growth factors, and extracellular matrix molecules. Mostly transcription factors regulate the patterning of cells from cranial neural crest, somites, and lateral plate mesoderm in the craniofacial, axial, and limb skeleton. From: (Olsen et al., 2000, p. 193).

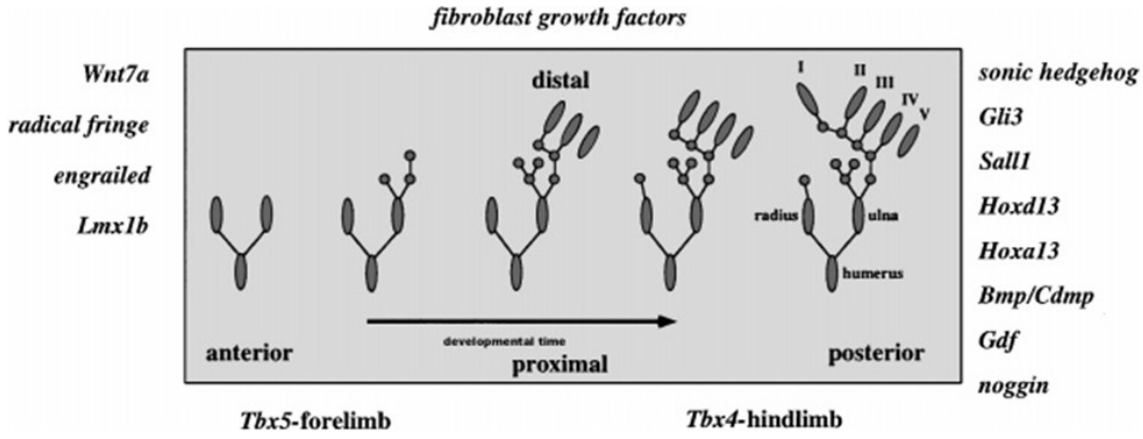


Figure 1-2: Diagram shows how cartilages in the developing forelimb are formed in a proximodistal sequence with increasing developmental time (arrow). The elements formed arise through a series of bifurcations and segmentations of mesenchymal condensations along the posterior (ulnar) axis of the limb. The limb genes are listed outside the diagram. On the right are genes controlled by sonic hedgehog and genes related to BMP signaling. At the top are genes expressed by the apical ectodermal ridge; at the bottom are transcription factor genes associated with establishment of limb identity. On the left are genes associated with specification of dorsal-ventral properties. From: (Olsen et al., 2000, p. 198).

A recent study by Quarto and co-workers has also demonstrated that frontal bone derived from neural crest displays a higher potential for osteogenic differentiation and higher capacity to undergo osseous healing compared with calvarial bone derived from paraxial mesoderm (Quarto et al., 2010).

The presence of neural crest-derived stem cells (NCSCs) within different adult craniofacial tissues such as the skin, periodontal ligament and the lamina propria of the palate, oral cavity and nasal turbinates indicates that these cells can differentiate into many cell types (Figure 1-3) (Kaltschmidt et al., 2012). Interestingly, the persistence of cell plasticity into adulthood could explain the regenerative potential of many mammalian craniofacial tissues (Kaltschmidt et al., 2012).

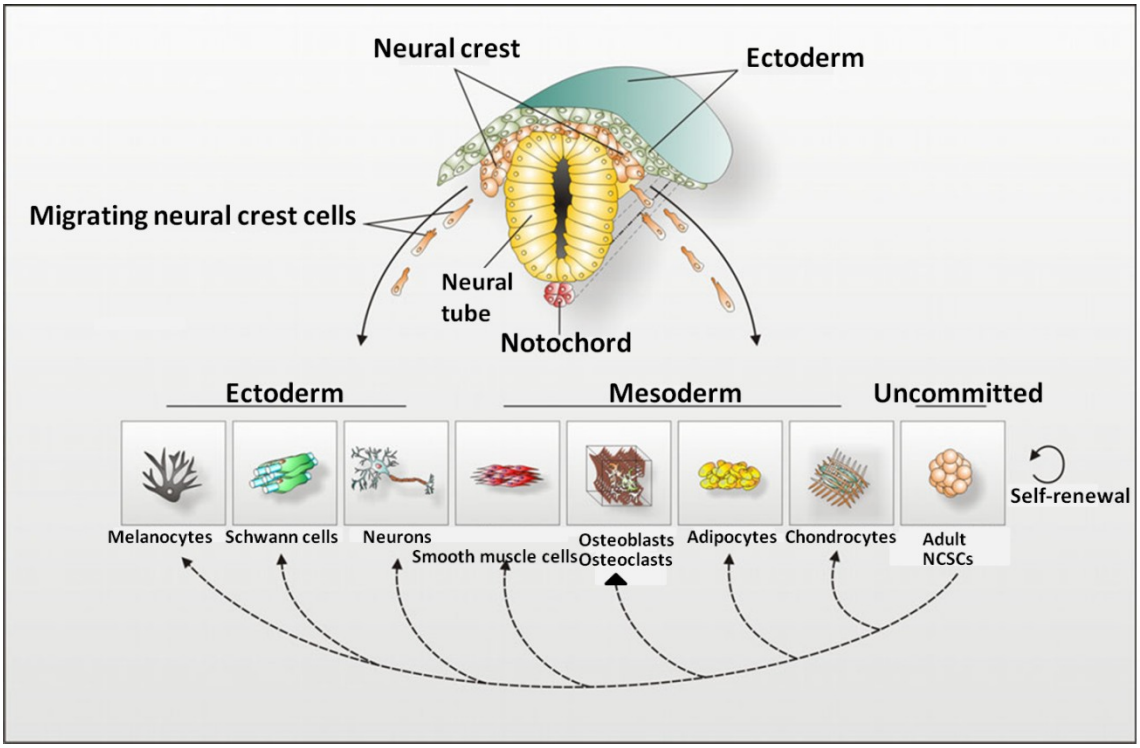


Figure 1-3: During the process of development in vertebrate embryo, neural crest cells migrate out between the newly formed ectoderm and the neural tube. The neural tube gives rise to a number of specialized cells such as ectodermal neurons, melanocytes, Schwann cells, mesodermal osteoblasts, adipocytes and smooth muscle cells. The new data suggest that embryonic neural crest-derived stem cells (NCSCs) not only contribute to terminally differentiated tissues, but also persist as uncommitted adult NCSCs which can undergo self-renewal, as well as multi-lineage differentiation. From: (Kaltschmidt et al., 2012, p. 659)

1.4 Osteoblast lineage

Bone is composed of four cell types. They are osteoblasts, osteocytes and bone lining cells which are derived from mesenchymal origin (Figure 1-4), and osteoclasts which are derived from a haemopoietic origin. The osteoblast lineage includes pre-osteoblasts, osteoblasts or mature osteoblasts, bone-lining cells and osteocytes (Long, 2012; Singer and Caplan, 2011).

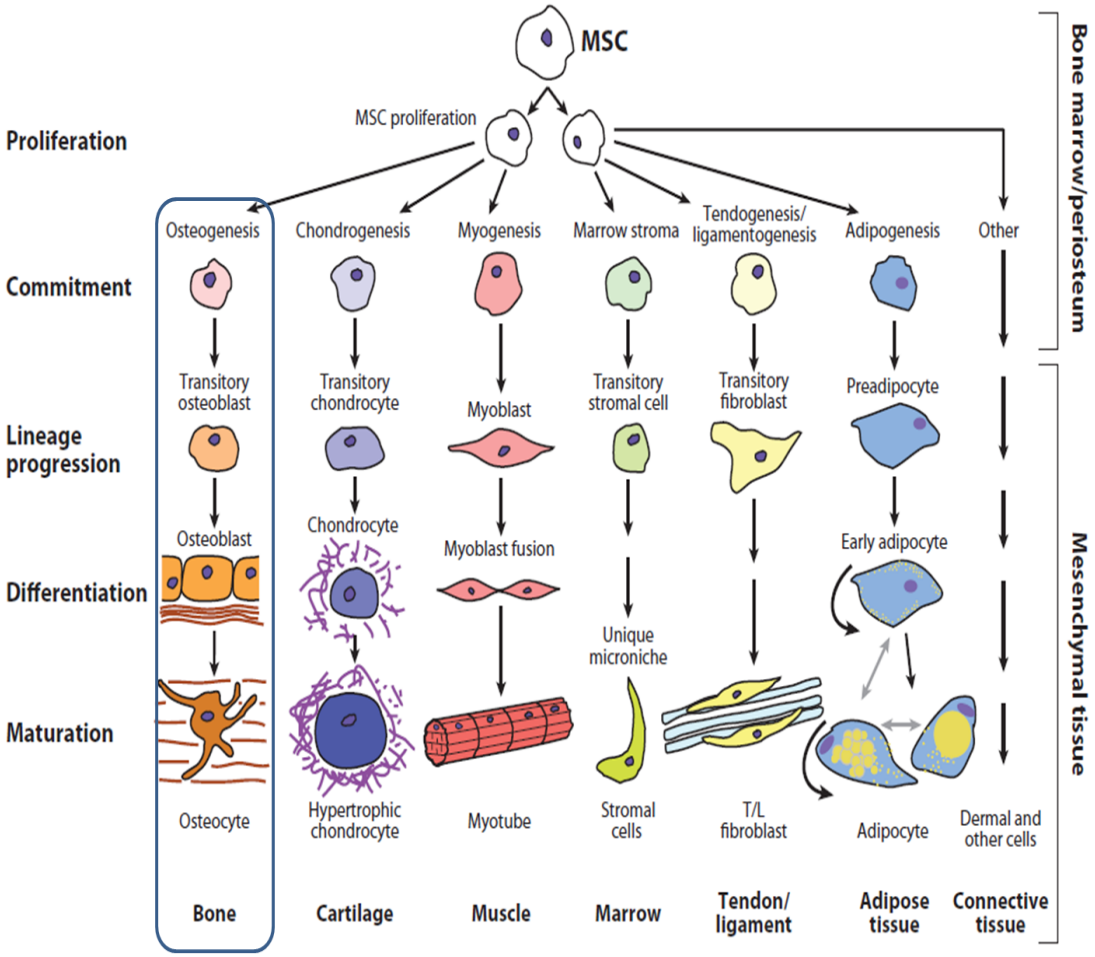


Figure 1-4: The mesengenic process of human mesenchymal stem cells (hMSCs) may develop into bone, muscle, or adipose tissue depending on the stimuli. Adapted from: (Singer and Caplan, 2011, p.458).

Osteoblasts

Osteoblasts are specialised cells responsible for bone formation and bone maintenance. They are derived from multipotent mesenchymal stem cells which may then undergo a sequence of proliferation and differentiation stages before expressing recognisable specific-bone markers, producing the organic matrix of bone, regulating mineralisation and regulating bone remodelling (Hughes et al., 2006).

The periosteum consists of two layers e.g. fibroperiosteum on the outside most distant from the bone surface, and an inner osteogenic periosteum where the pre-osteoblast, a directly recognisable cell of the osteoblast lineage, is found (Hughes et al., 2006). Pre-osteoblasts retain some proliferative capacity, but express many proteins associated with the mature osteoblast phenotype, including alkaline phosphatase and osteopontin (Owen, 1970). The other layer, the mature osteoblast layer, lies immediately adjacent to the bone (Hughes et al., 2006).

Osteoblasts are cuboidal or slightly elongated cells. They show the features of protein-producing cells containing rough endoplasmic reticulum, Golgi apparatus, secretory granules and microtubules in cytoplasm (Marks and Odgren, 2002; Sodek and McKee, 2000). Other organelles such as mitochondria, endosomal/lysosomal elements and cytoskeleton are also present (Marks and Odgren, 2002).

Osteoblasts are polarised cells which secrete matrix towards the bone surface (Marks and Odgren, 2002). They can release collagen filaments which assemble extracellularly into fibrils to form the osteoid or unmineralised bone matrix (Marks and Odgren, 2002; Sodek and McKee, 2000). The mature osteoblasts have an average 1-month lifespan, after which they undergo apoptosis and are replaced by newly differentiated osteoblasts or approximately one-third of these cells may be embedded into their deposited bone matrix and become osteocytes (Hughes et al., 2006).

Owen and co-workers (1990) hypothesised that genes responsible for production and deposition of extracellular matrix must be expressed during the proliferative period in order to initiate the onset of differentiation. This study provides the information on the three principle stages of osteoblast differentiation e.g. proliferation, matrix development and maturation, and mineralisation. A functional relationship has been described between the down-regulation of proliferation and the initiation of extracellular matrix maturation, based on stimulation of alkaline phosphatase and osteopontin gene expression when proliferation is inhibited (Owen et al., 1990).

The osteoblast lineage starts from mesenchymal stem cells through to osteocytes, the most differentiated cell of this lineage. Each transitional step requires the activation or suppression of signalling molecules to differentiate into osteoblast. Runx2, the major

transcription factor in osteoblastic differnetiation, is expressed in the late condensation stage of chondrogenesis and is substantially decreased in proliferating chondrocytes, but increases again in prehypertrophic and hypertrophic chondrocytes (Kronenberg, 2003). It is highly expressed in perichondrial cells and in osteoblasts and drives proliferating chondrocytes to differentiate further into hypertrophic chondrocytes(Kronenberg, 2003).

Runx2 is synthesised in chondrocytes, controlled by BMP2 and other regulatory factors, and causes a stage-dependent increase in the structural and functional proteins such as b-ALP, COLI, OP, ASP, BSP and OC. At a late stage, osteocytes become embedded in the mineralised matrix (Figure 1-5) (Wang et al., 2012).

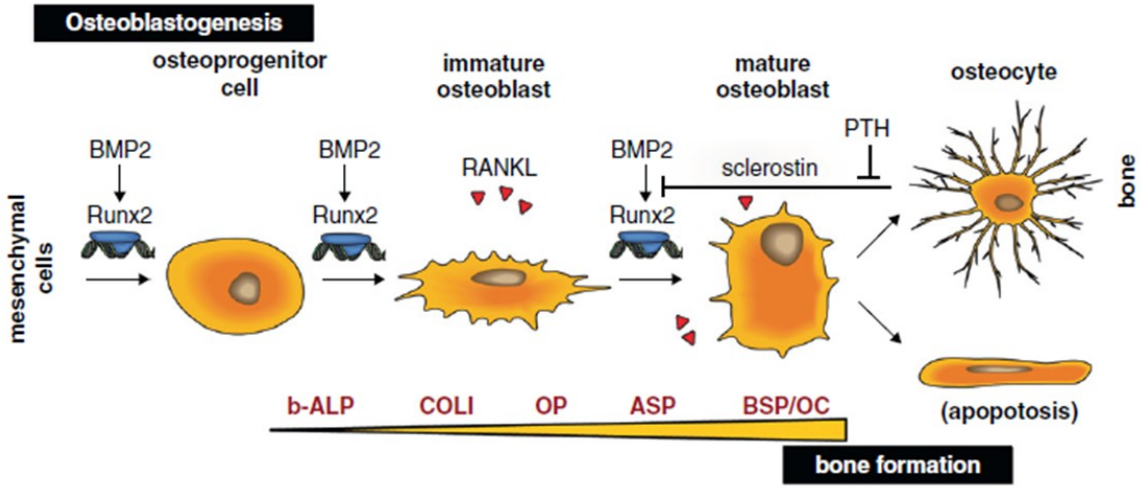


Figure 1-5: Osteoblastogenesis. Osteoblast differentiation starts from the mesenchymal stem cells to osteocytes at the end. From: (Wang et al., 2012, p. 572).

Osteoblasts derived from human mesenchymal stem cells can trans-differentiate into adipocytes and chondrocytes, and *vice versa* (Song and Tuan, 2004). In addition, fully differentiated osteoblasts, as shown by alkaline phosphatase (ALP) activity and osteocalcin synthesis, were able to undergo adipogenic differentiation after treatment with adipocyte inducing medium containing dexamethasone (Dex) and isobutylmethylxanthine (IBMX) (Nuttall et al., 1998), whilst osteoblasts were shown to transdifferentiate towards an adipogenic phenotype after 3 days of 18-α-glycyrrhetinic acid (AGRA) or oleamide-mediated inhibition of Gap-junctional communication (GJC) (Schiller et al., 2001).

Runx2 activates osteocalcin, an osteoblast-specific gene, expressed by fully differentiated osteoblasts (Ducy et al., 2000) and other transcription factors such as

OSX, MSX2, DLX5 and DLX6 also play important roles in the differentiation from a mesenchymal progenitor cell into a fully differentiated osteoblast (Stains and Civitelli, 2003), whilst PPARγ plays a key role in mediating adipocyte differentiation by decreasing Runx2 expression in osteoblastic cells (Liu et al., 2010).

In contrast, Homeobox-a2 genes (*Hoxa2*) act as negative regulators of Runx2 expression (Kanzler et al., 1998) and are also known to provide the second brachial arch with regional identity which inhibits bone formation in this area, possibly by preventing Cbfa1/Runx2 expression (Ducy et al., 2000), whilst Special AT-rich sequence-binding protein 2 (SATB2) inhibits expression of Hoxa2, but increasing the activity of Runx2 and Activating Transcription Factor 4 (ATF4) leading to osteoblast differentiation (Dobreva et al., 2006).

Based on studies of the limb skeleton, the developmental signals regulating key steps of mesenchymal progenitor cell differentiation to osteoblasts through distinct developmental stages are regulated by various developmental signals such as Hedgehog signalling, Notch signalling, WNT signalling, BMP signalling, and FGF signalling as recently reviewed by (Long, 2012) (See Figure 1-6).

During mammalian development, TGF-β/BMPs have essential roles in bone formation and exhibit useful regulatory functions in the body by signalling through both canonical Smad-dependent pathways and non-canonical Smad-independent signaling pathway (Chen et al., 2012). Bone morphogenetic protein (BMP) signalling stimulates osteoblast differentiation and function by the binding of BMP2 or BMP4 to their receptors resulting in phosphorylation of SMAD1, SMAD5 or SMAD8 which form a complex with SMAD4 and then enter the nucleus to regulate gene expression which eventually promotes the transition to RUNX2+OSX+ cells and enhances the function of mature osteoblasts (Long, 2012).

Fibroblast growth factor (FGF) signalling functions by binding to cell surface Tyr kinase FGF receptors and leads to the activation of multiple signalling modules by regulating pre-osteoblast proliferation, osteoblast differentiation, and the function of mature osteoblasts (Long, 2012). FGF also regulates chondrocyte proliferation and differentiation by shortening proliferative columns by decreasing chondrocyte proliferation directly and by suppressing Ihh expression (Kronenberg, 2003).

Indian hedgehog (Ihh) is a master regulator of bone development by coordinating chondrocyte proliferation, chondrocyte differentiation and osteoblast differentiation. Ihh is synthesised by chondrocytes during endochondral bone development and binds to Patched-1 (Ptc-1) which leads to activation of Smoothened (Smo), a membrane protein required for the cellular actions of Ihh. The active Smo triggers a cascade that leads to gene activation (Kronenberg, 2003). In the absence of Ihh, Runx2 was not expressed, but forced expression of Runx2 in the skeletogenic cells restored bone formation in the Runx2-null, but not in the Ihh-null embryo suggesting that Indian hedgehog requires additional effectors besides Runx2 to induce osteoblast differentiation (Tu et al., 2012). IHH binds to the receptor Patched homologue 1 (PTCH1), activates signalling through Smoothened (SMO), inhibits proteolytically cleaved GLI3 repressor (GLI3R), and promotes the full-length GLI2 activator (GLI2A), whilst derepression of GLI3R is sufficient to generate RUNX2+ cells. Both derepression of GLI3R and activation of GLI2A are necessary for progression to the RUNX2+OSX+ stage. The Hedgehog signalling through Indian hedgehog (IHH) is required for osteoblast differentiation through endochondral ossification (Long, 2012).

Notch signalling inhibits osteoblast differentiation by binding to their ligands Jagged (JAG) or Delta-like (DLL). Notch receptors are proteolytically cleaved by the γ-secretase complex, Notch intracellular domain (NICD) released from the plasma membrane and interacts with RBPJκ to activate downstream target genes, including HES (Hairy and Enhancer of Split) and HEY (HES-related with YRPW motif) family transcription factors and eventually inhibits osteoblast differentiation before OSX activation (Long, 2012).

WNT signalling promotes osteoblast differentiation by stabilisation of β-catenin followed by binding of WNT to its receptors Frizzled (FZD) and lipoprotein receptor-related protein 5 (LRP5) or LRP6 and leading to the transcription of β-catenin target genes and stimulating the RUNX2+ stage to the RUNX2+OSX+ stage, and from RUNX2+OSX+ cells to mature osteoblasts. In addition, WNT can also independently signal β-catenin through protein kinase Cδ (PKCδ) and promote the RUNX2+OSX+ stage through an unknown mechanism (Long, 2012).

The developmental signals regulating osteoblast differentiation are summarised in the schematic diagram (Figure 1-6) (Long, 2012).

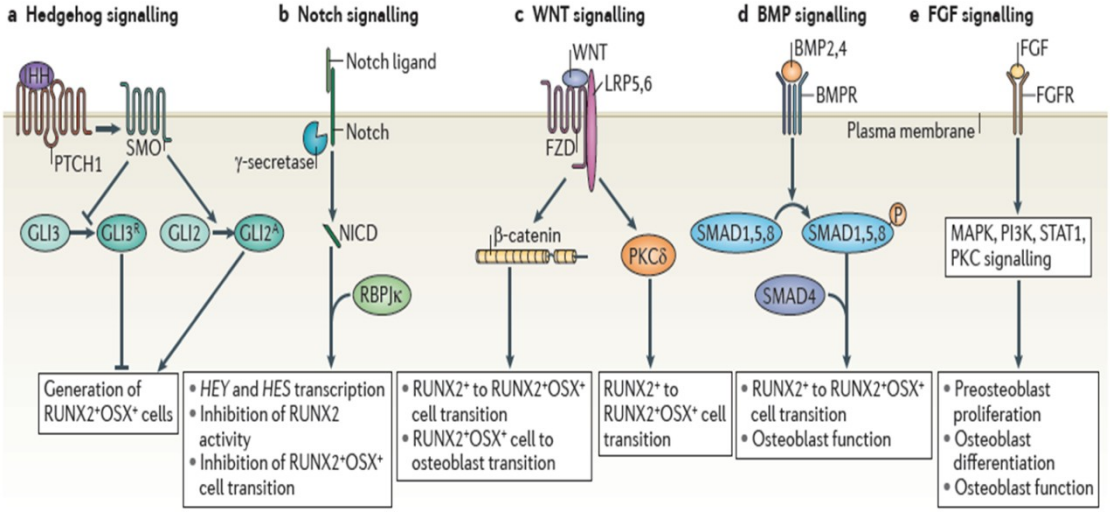


Figure 1-6: Developmental signals regulate osteoblasts to differentiate from mesenchymal progenitors through distinct developmental stages which regulated by various developmental signals; (a) Hedgehog signalling through Indian hedgehog (IHH) which binds to the receptor Patched homologue 1 (PTCH1) and activates signalling through Smoothened (SMO); (b) Notch signalling by binding to their ligands and leading to inhibition of osteoblast differentiation before OSX activation; (c) WNT signalling promotes osteoblast differentiation; (d) Bone morphogenetic protein (BMP) signalling stimulates osteoblast differentiation and function by the binding of BMP2 or BMP4 to their receptors results in phosphorylation of SMAD1, SMAD5 or SMAD8; (e) Fibroblast growth factor (FGF) signalling by binding to cell surface Tyr kinase FGF receptors (FGFRs; FGFR1–FGFR4 in humans and mice) and leading to the activation of multiple signalling modules. From: (Long, 2012, p. 32).

Osteocytes

Osteocytes are mature osteoblasts embedded within the bone matrix. They are the most abundant cell type found in bone, consisting of 90-95% of all bone cells in the adult skeleton (Bonewald, 2007). They may have roles in the maintenance of bone mass, regulating osteoclastic resorption, blood-calcium phosphate homeostasis and modulating responses to mechanical stress. In general, they are responsible for maintenance of the skeleton by regulating both matrix synthesis and resorption. However, the mechanisms of how osteoblasts become osteocytes remain unclear (Franz-Odendaal et al., 2006). Each osteocyte in lacunae extends cytoplasmic processes through canaliculi in the matrix to contact processes of adjacent osteocytes and through gap junctions with osteoblasts on the bone surface for their communication. The canalicular-lacunar system is essential for cell metabolism to diffuse nutrients and waste products and may be important for signal transduction due to mechanical loading and provide access to blood vessels (Bonewald, 2007).

Bone lining cells

Bone lining cells are flattened and found on bone surfaces when they become quiescent. They are inactive osteoblasts which show decreased protein secretion and undergo a process of morphological and functional changes. The bone lining cells may be able to regain full capacity to produce bone matrix. Bone lining cells and osteocytes appear to form an extensive homeostatic network and regulate plasma calcium concentration (Parfitt, 1989).

Osteoclasts

Osteoclasts are multinucleated cells derived from cells of the monocyte/macrophage lineage which fuse together. They are responsible for bone resorption and regulate bone remodelling processes with osteoblasts (Hughes et al., 2006; Teitelbaum, 2000). The ability of the osteoclast to degrade bone depends on a tight attachment to the bone surface together with ions and matrix-degrading enzymes secreted into the sealed lacunae, break down the inorganic and organic matrices, thus releasing bone-derived factors including Ca2+ ions and matrix proteins such as transforming growth factor-β and osteocalcin into the marrow cavity (Edwards and Mundy, 2011).

Bone cell types and their functions are summarised in Table 1-1.

Cell type	Morphological characteristics	Function
Osteoblasts	cuboidal shaped	- synthesis and regulation of bone ECM deposition and mineralization - respond to mechanical stimuli
Osteocytes	stellate shaped	- calcification of osteoid matrix - blood-calcium homeostasis - mechanosensor
Bone lining cells	flat shaped	- may be able to regain full capacity to produce bone matrix - homeostatic network with osteocytes
Osteoclasts	multinucleated cells	- bone resorption

Table 1-1: Summary of bone cell types and their functions. Modified from: (Salgado et al., 2004).

1.5 Regulatory signals

There are many extracellular signalling molecules which may participate in the regulation of osteoblast differentiation and bone formation. These include locally acting growth factor molecules and systemic hormones, some of which are reviewed briefly below.

1) Local regulators of osteoblasts

A wide range of locally acting growth factors has been shown to be able to regulate osteoblast function. These include the BMPs, wnts, hedgehog family, notch, FGFs, PDGF, IGFs, TGFß and EGF. These have been reviewed by Hughes and co-workers (Hughes et al., 2006). Some of these signalling molecule families which particularly influence early stages of osteoblast differentiation include the BMPs, wnts, and hedgehog.

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor-beta (TGF-β) superfamily are multi-functional growth factors (Hughes et al., 2006). BMP signals not only shape tissues ranging from embryonic patterning through stem cells and their niches, they also regulate stem cell fate and are involved in tissue regeneration throughout the whole body (Wagner et al., 2010).

BMP was first identified and named "bone morphogenetic protein" by Urist in 1965 based on their ability to induce the full cascade of endochondral ossification when implanted to ectopic sites such as rat muscle (Urist, 1965). Up to 20 BMP family members have now been identified, characterized and their properties have been extensively further studied (Chen et al., 2004) such as their induction of BMSCs to become osteoblasts, promote osteoblast differentiation and induce orthotopic and heterotopic bone *in vitro* and *in vivo*. The immediate downstream molecular targets of BMP receptor binding are Smad1, 5 and 8 and these play an important role in BMP signal transduction and postnatal bone formation (Wagner et al., 2010).

Wnts are a large family of at least 19 secreted glycoproteins that trigger multiple signalling during embryonic development. The effects occur in cells *via* canonical or non-canonical pathways through binding to the Frizzled (Fzd) receptor family members (Galli et al., 2010; Monroe et al., 2012). Stages of differentiation are indicated by transcription factors expression e.g. Prx1, Runx2, osterix (Osx), and osteocalcin (OCN) at low or at high levels. *Wnt* signaling promotes the early phases of commitment, differentiation, and the expression of osteoprotegerin (OPG), but inhibits high levels of osteocalcin expression. In addition Sclerostin (Scl) may inhibit *Wnt* signaling in a negative feedback loop (Galli et al., 2010; Monroe et al., 2012) (Figure 1-7).

Low-density lipoprotein receptor-related proteins (Lrp) are evolutionarily conserved plasma membrane receptors with many functions including lipid metabolism, cargo transport, and cellular signalling. Lrp5/6 are low affinity co-receptors for Wnts and high affinity receptors for soluble Wnt antagonists: Scl, Sost-dc1, and Dkk1. Lrp4 is also emerging as a regulator of bone mass density (Monroe et al., 2012). Wnt/LRP5/b-catenin pathway (Low-density lipoprotein receptor Related Protein 5) also plays a critical role in promoting osteoblast differentiation and maintaining bone mass (Li and Xiao, 2007).

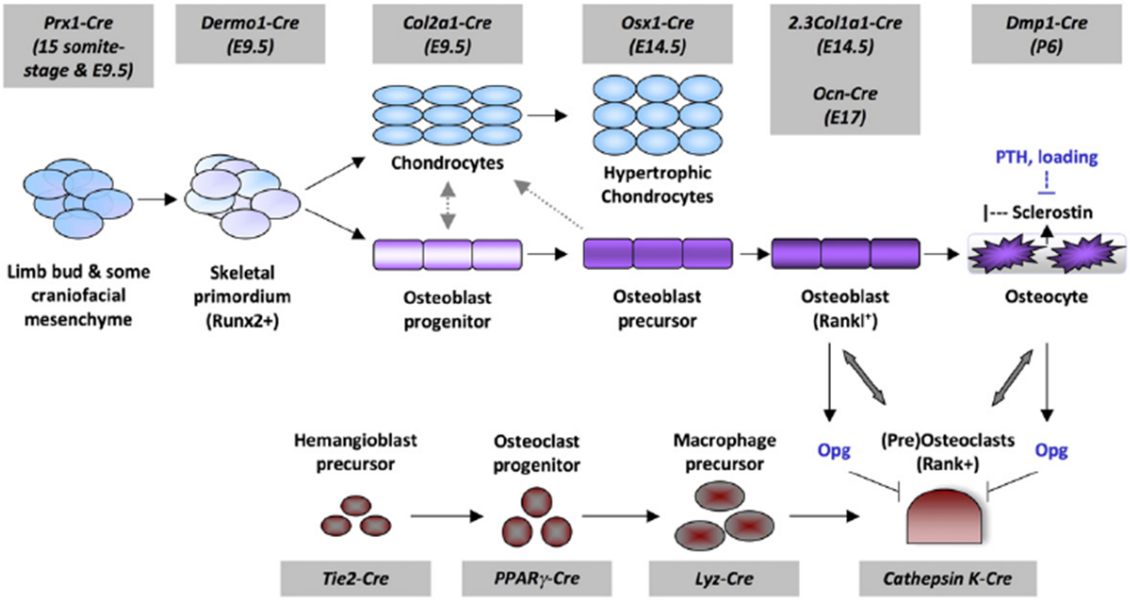


Figure 1-7: Signalling involved in cell lineage in bone development. Mesenchymal progenitor cells give rise to osteoblasts and chondrocytes, whereas hematopoietic precursors give rise to osteoclasts. At different stages of their maturation, various promoters can be used to drive transgene or Cre expression in these cells. Mature osteoblasts and osteocytes stimulate osteoclast maturation through RANKL/RANK/OPG system. In addition, osteocytes secrete Scl to inhibit Lrp5 activities, but Scl production is suppressed by PTH and mechanical loading to increase bone formation. From: (Monroe et al., 2012, p. 6).

Hedgehog (Hh) signalling is involved in regulating verterbrate osteoblastogenesis such as digit growth. There are three Hh proteins in the mammalian genome namely sonic Hedgehog (*Shh*), Indian Hedgehog (*Ihh*) and desert Hedgehog (*Dhh*) (Dorus et al., 2006). Disruption of the Hh signalling pathway resulted in failure of cells to undergo osteoblast differentiation (Long et al., 2004).

Fibroblast Growth Factor-2 (FGF-2) supported osteogenic precursors cells derived from human bone marrow for their expansion and maintenance (Martin et al., 1997). However, FGF-2 will be further discussed in Chapter 3.

2) Systemic regulation of osteoblasts

There are many systemic hormones that have roles in osteoblast regulation as summarised in Table 1-2. However, our particular interest is the role of oestrogen in regulating bone, for example as is seen in postmenopausal osteoporosis.

Oestradiol is transduced by two receptors belong to the superfamily of nuclear receptors known as oestrogen receptor (ER)-α or and ER-β (according to the Nuclear Receptors Nomenclature Committee; NR3A1 and NR3A2, respectively) (Bonnelye and Aubin, 2005; Bonnelye et al., 2011). The nuclear receptors are transcription factors comprising of both ligand-dependent molecules such as steroid hormone, thyroid hormone, retinoic acid, vitamin D receptors, and a large number of so-called orphan receptors (Bonnelye and Aubin, 2005).

Postmenopausal osteoporotic bone loss is due to oestrogen deficiency resulting in decreased bone formation with increased of bone turnover (Turner et al., 1994). Lajeunesse et al. (1994) examined the effect of 17β-oestradiol on the human osteosarcoma cell line MG-63. They found that 17β-oestradiol upregulates osteocalcin secretion and alkaline phosphatase synthesis in a dose-dependent manner (Lajeunesse, 1994). In addition, they also suggested that oestrogens may regulate bone remodelling by modulating hormonal-induction of protein involved in bone mineralisation. This is an indirect effect as it did not modify basal activities, instead involving a regulation of $1,25(OH)_2D_3$ receptor level in MG-63 cells.

Oestrogen and parathyroid hormone (PTH) enhance *Runx2* expression and activity through anabolic effects, however, oestrogen negatively regulates *Runx2* in osteoclastogenesis (Komori, 2008).

Hormones	General Functions	Specific Functions	References
Oestrogens	-regulators of bone mass	-receptors activation leading to specific gene transcription	(Liedert et al., 2010)
Progesterone	-bone metabolism	-effect osteoblast proliferation and differentiation	(Schmidt et al., 2000)
Androgen	-skeletal development and maintenance of bone mass in men and women	-influence growth plate maturation and closure	(Wiren et al., 2002)
PTH, PTH1R	-maintenance of calcium homeostasis	-promote calcium release from bone -Enhance kidney calcium reabsorption	(Hock, 2002)
Growth Hormone	-longitudinal bone growth	-enhance bone remodelling	(Karaplis, 2002)
Thyroid Hormone	-skeletal development and differentiation	-stimulate osteoblast proliferation, -promote differentiation, -increase alkaline phosphatase activity, - osteoclast differentiation	(Stern, 2002)
Glucocorticoids and other steroids	-regulate bone metabolism	-differentiation and function of osteoblasts and osteoclasts	(Kream and Lukert, 2002)
Insulin	-influence the physiology of cartilage and bone	-stimulate endochondral bone growth and osteoblast proliferation and function	(Verhaeghe and Bouillon, 2002)
Vitamin D	-development and maintenance of bone	-bone calcium and phosphate homeostasis	(Norman, 2002)
Leptin	-may control bone metabolism	-controlling bone mass	(Lerner, 2002)

Table 1-2: Summary of hormones that may regulate bone and osteoblasts

3) Transcription factors regulating osteoblast function

Both local and systemically acting extracellular signalling molecules act ultimately by ligand binding and modulating cell gene expression. There is now considerable information on a range of transcription factors which appear to have important roles in regulating osteoblast differentiation and function.

The essential regulators of osteoblast lineage cells are the core binding protein from the runt family of transcription factors (Runx2) and osterix (OSX). Runx2 is a major regulator of bone formation and its expression is absolutely required for osteoblast differentiation. Osterix is a downstream target of Cbfa1/Runx2 and required for normal osteoblast differentiation as demonstrated in KO-mice study (Nakashima et al., 2002). The mammalian *Runx* (*runt-related transcription factor x*) genes encode a family of transcription factors; including *Runx1*, *Runx2*, and *Runx3*, are homologous to

Drosophila runt. Multiple signalling pathways regulate *Runx* gene expression and protein function at multiple levels (Ducy et al., 1997).

Runx2, a member of a family of transcription factors which shares the DNA binding domain of the *Drosophila* pair rule gene *runt* (Kronenberg, 2003), is an osteoblast-specific transcription factor and a regulator of osteoblast differentiation (Ducy et al., 1997) which directs mesenchymal cells to differentiate into osteoblasts and inhibits adipocyte differentiation (Komori, 2006). The expression of bone matrix protein genes such as *Col1a1*, *Spp1*, *Ibsp*, *Bglap*, and *Fn1* were upregulated by RUNX2 during osteoblast differentiation *in vitro* and many promoters such as *Col1a1*, *Col1a2*, *Spp1*, *Bglap*, and *Mmp13* were also activated (Komori, 2010). Importantly, RUNX2 induces osteoblast differentiation and increases the number of immature osteoblasts, whilst *Runx2* expression must be down regulated for differentiation into mature osteoblasts to occur to form mature bone (Komori, 2010).

Runx2 expression in bipotent progenitor cells differs in skeletal elements that ossify by the intramembranous route from those elements that ossify by the endochondral route. The transition to functional osteoblasts expressing high levels of osteoblast marker genes requires *Osx*. This transcription factor cooperates with *Runx2* to activate bone-specific genes and inhibit *Sox9* expression. Thus, preventing the cells from entering the chondrocyte differentiation pathway (Owen et al., 1990).

Msx2 (mesh-less 2) is a mammalian homologue of *Drosophila* muscle segment homeobox gene (*msh*) (Li and Xiao, 2007). *Msx2* regulates early osteoblast development (Schinke, 2002), whilst *Dlx5*, a homologue of the Distal-less (Dll) in *Drosophila*, is a BMP-inducible homeoprotein which induces the transcriptional activity of osteoblast differentiation.

The transcriptional properties of *Msx* and *Dlx* proteins display reciprocal actions where *Msx2* acts as repressor of transcription and is essential for normal ossification (Marie, 2008), whilst *Dlx5* acts as a transcriptional activator (Schinke, 2002). *Msx1* and *Dlx5* synergistically regulate osteogenesis during frontal bone development particularly in closure of the suture (Chung et al., 2010). Studies on primary culture of chick calvarial osteoblasts revealed that *Msx2* stimulates cells proliferation, but suppress differentiation

via repress *Runx2* transcription activity (Li and Xiao, 2007). Cellular and genetic analyses showed that several transcription factors that belong to homeobox proteins (*Msx1*, *Msx2*, *Dlx5*, *Dlx6*) may play a role in osteoblast differentiation.

Iroquois related homeobox genes (*Irx*) 5 and 6, a family of homeodomain-containing transcription factors involved in patterning and regionalisation of embryonic tissues; *Irx5* expressed in an osteocyte like cell line (Paic et al., 2009).

T-box (Tbx) 3, a transcriptional repressor that regulates osteoblast proliferation, its expression increases during osteoblast differentiation (Govoni et al., 2009). The mutation of *Tbx3* results in limb malformation, but transgenic overexpression of *Tbx3* in osteoblasts can promote proliferation, but suppress differentiation and may involve in regulating key transcription factors in osteoblast differentiation (Govoni et al., 2009).

A summary of transcription factors that may have specific roles in regulating osteoblast function is shown in Table 1-3.

Transcription Factors	Functions	References
Runx2 or Cbfa1	osteoblast differentiation	(Ducy et al., 1997; Schinke, 2002)
Msx2, Dlx5	osteoblast differentiation	(Schinke, 2002)
Irx5	embryonic patterning and regionalisation	(Paic et al., 2009)
Tbx3	regulates osteoblast proliferation	(Govoni et al., 2009)
Osx	osteoblast differentiation	(Nakashima et al., 2002)
Taz	osteoblast differentiation act on bipotent osteoblast and adipocyte stem cells	(Hong et al., 2005)
Fra-1	regulate bone mass by osteoblasts and chondrocytes through bone matrix production	(Eferl et al., 2004)
deltaFosB	overexpression increases bone formation and inhibits adipogenesis	(Sabatakos et al., 2000)
c-Abl	osteoporotic and defects in osteoblast maturation in *Abl* deficient mice	(Li et al., 2000)
RORα	direct modulation of a bone matrix component	(Meyer et al., 2000)

Table 1-3: Summary of some important transcription factors that regulate bone and osteoblasts

1.6 Regional specification of spatial and positional identity

As previously discussed, differences in functional characteristics of similar cell phenotypes might be determined by their embryological origins and their anatomically diverse positions within the mature organisms. Body morphogenesis in development and evolution is regulated by *Hox* genes. Therefore, mutations in *Hox* genes transform one body segment into another (Wolpert, 1994). In mammals the *Hox* genes, which were first discovered in fruit flies, comprise 39 transcriptional factors and are grouped into four clusters arranged on different chromosomes (Iimura and Pourquie, 2007). *Hox* genes encode transcription factors that regulate body formation during development.

They belong to a homeotic genes family called the homeobox which characterised by approximately 180 base pairs of a DNA sequence and codes for the homeodomain, about 60 amino acid residues in DNA-binding domain.

Hox genes have essential roles in controlling time and route of body development to identify embryonic segment, different *Hox* genes being expressed in different segments. Vertebrates have four distinct *Hox* genes clusters, called A, B, C and D, which are involved in similar developmental processes (Iimura and Pourquie, 2007). Their spatial pattern of expression along the anterior-posterior and proximal-distal axes of the embryo reflected by their order on the chromosomes called colinearity (Rinn et al., 2008b).

The positional identity of skin by "*HOX* code" may dictate and influence site-specific epidermal differentiation (Chuong, 1993). For example, expression of *HOXA13* is required to maintain epidermal inductive properties of distal fibroblasts (Rinn et al., 2008a). The evidence in *HoxA13* knockout limb showed that *Wnt5a* expression is substantially reduced, but not completely absent and also expressed more proximally than the *HoxA13* expression domain suggesting that other transcription factors also directly induce *Wnt5a* expression via more distant enhancer sequences or indirectly via HOXB6 (Rinn et al., 2008a). In this context, *Wnt5a* is specifically expressed in distal fibroblasts.

The polarity of the embryo, formation of the anterior-posterior body axis, and body segmentation are controlled by *Hox* gene clusters during embryogenesis in all species. Specific *Hox* genes are expressed and demarcate different positional identities that lead to cellular differentiation and tissue morphogenesis in site-specific manner.

The homeobox genes are expressed in a site-specific manner to specify spatial and positional information. Knocking out individual Hox genes in *Drosophila* causes homeotic transformations which one body part develops into another such as the Antennapedia mutant, in which legs develop on the fly's head instead of antennae suggesting that Hox genes control development of morphologically distinct regions in a segmented animal (Kaufman et al., 1990; Lewis, 1978).

The markers of regional development and bone metabolism are differentially expressed site-specifically. *Hoxa* gene expression patterns are one example of markers of embryologically determined "positional identity", although other *Hox* genes family members (*Hoxb*, *Hoxc* and *Hoxd* families) may be expressed in a co-linear fashion with *Hoxa* and also reflect embryological positional identity.

1.7 *Hoxa* genes and positional identity

1) Persistence of positional identity in the adult organism

Bones from different anatomical sites in the adult organism behave differently. For example, long bones are dependent on mechanical loading for maintenance of bone mineral density and are susceptible to osteoporosis, whereas calvarial bones are not subject to extensive mechanical loading, but still maintain a high bone mineral density and are generally not affected by osteoporosis. This raises the question as to whether the osteoblasts in these regions are pre-programmed to be phenotypically distinct because they retain their embryonic positional identities.

In a recent study from our lab, Rawlinson and co-workers investigated the expression and maintenance of positional identity markers in adult rat bones from different regions. Mouse Genome and Rat Genome GeneChips were used to compare gene expression profiles from long bones and parietal skull bones samples, and also on osteoblastic cells isolated from these bones (Rawlinson et al., 2009b). In that study 1,236 genes (approximately 4% of the genome) were found to be at least two-fold differentially expressed between ulna limb and parietal skull bone, and most remarkably, in cultured cells derived from ulnae and calvariae also showed significant differences in expression levels of 249 genes. Dendrograms generated by gene tree clustering analysis in GeneSpring 6.1 compared basal expression of all filtered genes in skull and limb bone organs and in cell cultures are shown in Figure 1-8 a-c.

Strikingly, amongst these differentially expressed genes were those associated with embryological positional identity including *Hox* genes, which were preferentially expressed in ulna cells, and functionally relevant osteoblast associated genes such as

Spp1 (osteopontin), Alcam (activated leukocyte cells adhesion molecule), Sfrp2 (secreted frizzled-related protein 2); Apo-E, (apolipoprotein E), Smoc2 (SPARC related modular calcium binding 2), and Fst, (follistatin).

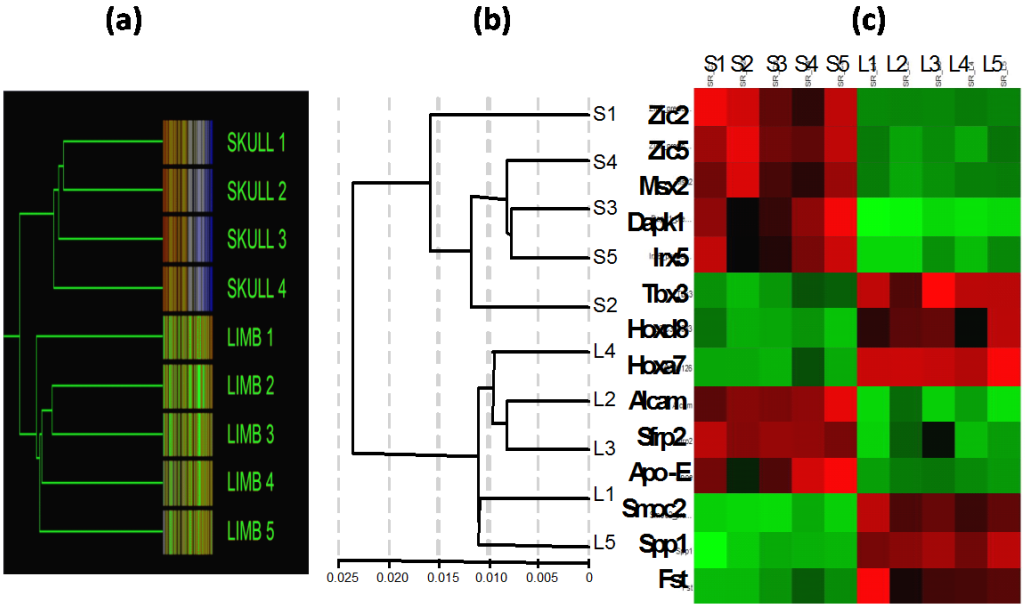


Figure 1-8: Bones from different skeletal sites represent distinct populations. (a) Using GeneSpring 6.1 for Gene tree clustering analysis to generate dendrogram compared all filtered genes expressed in skull and limb bone organs, (b) Dendrogram signified genotypic differences between skull and limb adult bone-derived cells (S-Bdc= skull bone-derived cells and L-Bdc= limb bone-derived cells) in 5 matched pairs, (c) Heat map for markers for regional development shows cultured S-Bdc (S1–S5) preferentially express genes associated with the craniofacial development of neural crest derived cells (red), whilst L-Bdc (L1–L5) preferentially express genes for limb development and patterning. Adapted from: (Rawlinson et al., 2009b, p. 2, p. 6).

In support of the idea that adult cells retain their embryological functional identity are studies that demonstrate similar findings in fibroblasts. Fibroblasts have been shown to have consistent and distinct site-specific variations in gene expression including expression of "positional identity" genes such as *Hoxa*. An experiment by Rinn and colleagues characterised the diversity of human fibroblasts testing from 47 primary cultured fibroblasts derived from 43 anatomical sites for their maintenance in site-specific differentiation (Rinn et al., 2006). These fibroblasts were cultured for at least 5 passages to remove the effect of their native environment and the microarray of gene expression was analysed. Their findings confirmed that site-specific variation in fibroblast gene expression was related to their positional identity with respect to anatomical regions; anterior-posterior, proximal-distal, and dermal-nondermal (Rinn et al., 2006).

The positional identity study of differentiated fibroblasts has demonstrated the stability after long-term passage of transcriptional patterns *in vitro*, suggesting that differentiated fibroblasts have mechanisms to maintain the epigenetic information which is important for anatomically specific differentiation as the embryonic pattern of HOX gene expression appears to be retained in diverse sampling of cultured adult fibroblasts (Rinn et al., 2006).

2) Evidence that "positional identity" may be reprogrammed

In Drosophila, Hox gene expression is regulated by Polycomb group (PcG) and trithorax group (trxG) proteins, which are also called maintenance proteins (MPs). Thus, the interplay between PcG and TrxG/MLL complexes is essential for proper maintenance, silencing and activation of Hox and many other genes in vertebrates (Soshnikova and Duboule, 2008). H3K27me3/me2 at Hox promoters was removed by a new family of histone demethylases for induction of Hox gene transcription during differentiation of totipotent embryonic stem cells (Soshnikova and Duboule, 2008).

The retention of positional identity in adult differentiated cells might contribute to its faithful homeostasis but may also limit its plasticity, leading to a loss of regenerative ability in higher vertebrates (Wang et al., 2009). In support of this concept Leucht et al. studied the effect of transplanting tibial and mandibular skeletal stem cells into bony defects in mandibles and tibia (Leucht et al., 2008a). They used transgenic mice using GFP-labelled cells to isolate cells, and follow them when re-implanted into the defects. The experiment was designed to have 4 different groups e.g. tibia-Hox+ve cells placed into mandibular-Hox-ve recipient site, mandibular-Hox-ve implanted into tibia-Hox+ve recipient site in order to mimic mismatch grafting, whilst the other two groups were matched Hox-expressed cells grafts e.g. tibia-Hox+ve cells placed into tibia-Hox+ve site and mandibular-Hox-ve cells placed into mandibular-Hox-ve sites for controls (Leucht et al., 2008a). The results showed that Hox+ve cell grafts were not able to contribute to the repair of a Hox-ve injury site. In contrast, Hox-ve grafts when transplanted into a Hox+ve injury site were able to contribute to bone regeneration. They concluded that Hox-ve skeletal stem cells can only heal orthotopic Hox+ve bone injury site (Figure 1-9).

Furthermore using *in situ* hybridization, they showed that mandibular-Hox-ve cells when transplanted to the (*Hoxa* +) tibia exhibited *Hoxa11* expression. These results strongly suggest that the neural crest-derived *Hoxa*- cells showed greater plasticity and could be reprogrammed by some aspect of their local environment to express the positional identity of the location of the recipient site. In contrast, *Hoxa* positive cells continued to express their original positional identity, were not reprogrammed by transplantation and hence were not able to participate in the healing process of the recipient site.

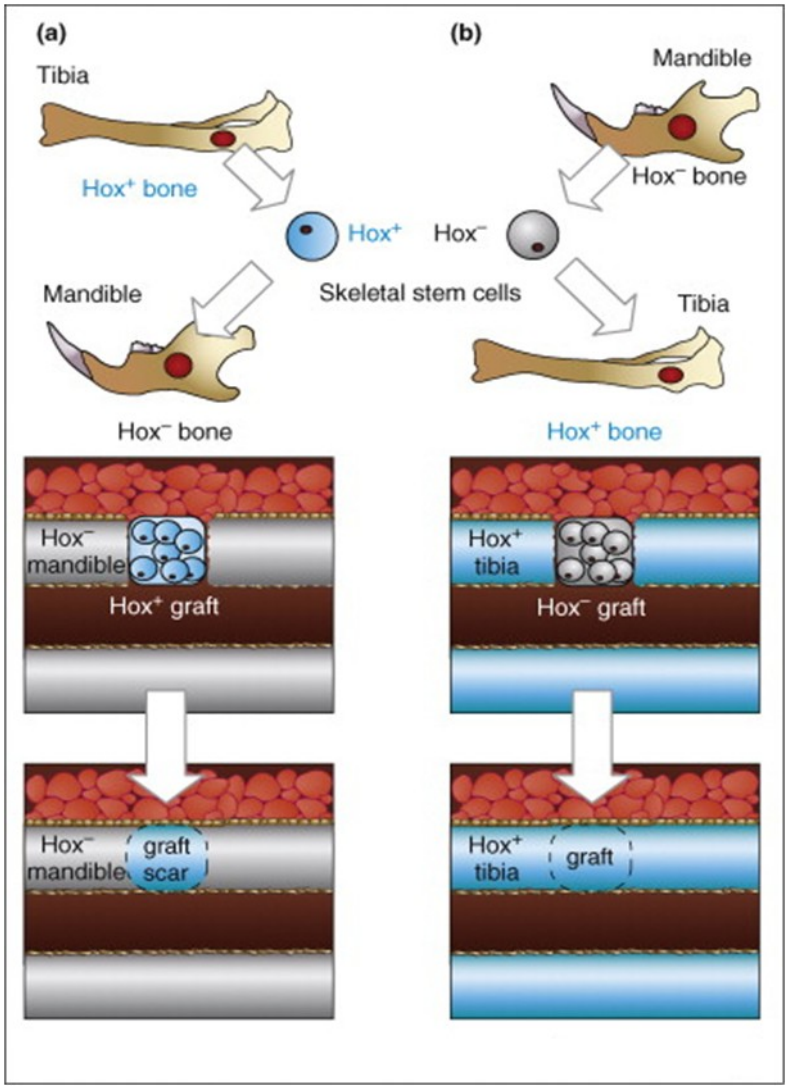


Figure 1-9: Hox status and bone regeneration in mouse. Cells expressing *Hox* genes are Hox+ and not expressing *Hox* are Hox-. Hox+ cells can only heal orthotopic Hox+ bone injury site (tibia). (a) Hox+ cells cannot repair a Hox- injury site (mandible). (b) On the other hand, Hox- cells will express the ectopic *Hox* gene when transplanted into a Hox+ injury site and regenerate the ectopic bone. From: (Wang et al., 2009, p. 273).

In support of the idea of reprogramming of positional identities in the studies of fibroblast positional identity described by Rinn and co workers in 2008, they suggested that fibroblasts may be reprogrammed by co-culture and physical contact between heterotypic fibroblasts (Rinn et al., 2008a). In addition, a co-culture study of primary dermal fibroblasts derived from palms or soles described by Yamaguchi and co workers suggested that this co-culture could reprogramme keratinocytes from other sites to express acral specific differentiation genes, such as Keratin9 (K9) (Yamaguchi et al., 1999).

3) Role of Hox in regenerative capacity

To date, data suggest that *Hoxa* genes, and other genes associated with positional information in the embryo, have a role in specifying transcriptional memory which continues into the adult organism. This may limit their plasticity and bone regenerative potential. A clinical implication of these findings is that this may lead to improved success rate of grafted cells which could be influenced by distinguishing one type of adult stem or progenitor cell from another. Plasticity of positional identity may be important in wound repair when cells need to integrate in the wound bed for appropriate regeneration (Leucht et al., 2008b). Therefore, different locations of donor-sites for bone grafting may need to be taken into consideration for bone grafting procedures as these molecular differences may have an effect on bone regenerative capacity and the clinical outcome. Although the data do suggest the possibility that positional identities might be reprogrammed, at least in some circumstances, there is little information on how these cells might be modulated during bone healing after grafting especially at the molecular level.

Questions still remain about the mechanism and further studies would be needed to directly test whether adult stem cells actually retain a Hox code, and then whether these potential molecular differences are crucial features of successful tissue repair and regeneration. This concept of cells regaining their plasticity which was found earlier in development and still be observed in induced pluripotent stem cells may increase our understanding of these mechanisms, perhaps by using cell modulation.

1.8 Positional identity: implication that bone cells are not all the same

The nature of the signals leading to the diversity of osteoblast gene expression profiles is not known (Aubin, 2002). Our recent evidence suggests that genes associated with mineral density and bone mass are differentially expressed in distinct skeletal sites. Our observations for the first time reveal the developmental gene expression-based "positional identity" in the adult skeleton (Rawlinson et al., 2009b).

We think of osteoblasts as bone forming cells, but this recent evidence suggests that osteoblasts from different sites are distinct. Therefore, osteoblasts may be pre-programmed and show phenotypic differences between different sites or origin. Those findings mentioned in the literature review have raised interesting questions whether adult osteoblastic cells derived from different sites can retain their stability of *Hoxa* gene expression, transcription factors and osteoblast-associated genes *in vitro*, and whether mimicking grafting procedure by using heterotypic culture system *in vitro* may have an effect on bone cell phenotype, their osteogenic potentials and regenerative capacity. The hypothesis and process of this study are summarised below (Figure 1-10).

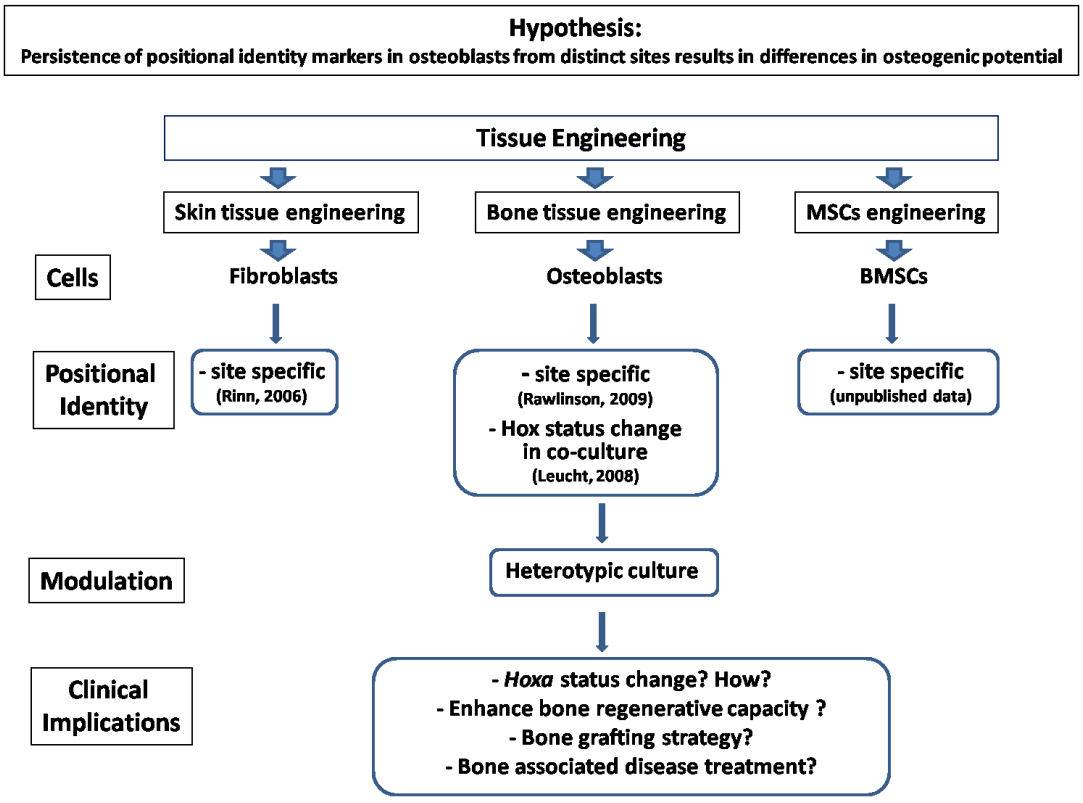


Figure 1-10: Hypothesis to test whether heterotypic culture may have an effect on different *Hoxa* genes expressed osteoblastic cells.

1.9 Aims

The overall aims of the study are to test the hypothesis that persistence of positional identity markers in osteoblasts from distinct sites results in differences in osteogenic potential.

Specific objectives of the proposed studies include:

To investigate the stability of positional identity markers in isolated rat osteoblasts *in vitro* and in heterotypic culture of cells derived from distinct regions and expressing different *Hoxa* gene profiles;

To establish a cell culture model derived from different sites, grown together in heterotypic culture and sorted by flow cytometry to address whether site-specific gene expression may be reprogrammed by heterotypic culture between Hox- and Hox+ cells;

To determine if osteoblasts derived from distinct sites are phenotypically different;

To investigate if positional identity of osteoblasts may influence their bone regenerative capacities constitutively and in response to specific hormone or growth factor stimulation.

Chapter 2

Chapter 2: General materials and methods

2.1 Introduction

This chapter describes general methods used in this study. Specific experimental details are described in the appropriate chapters.

2.2 Culture of primary cells

All protocols for the use of animals followed Queen Mary University of London (QMUL) and King's College London (KCL) regulations for animal research and in accordance with Home Office protocols.

2.2.1 Establishing adult mice osteoblastic cells using collagenase isolation method

In the first experiment for establishing osteoblast primary cell cultures, 5 male mice (weigh 22.04 g±0.74) aged 47-54 days were obtained. The mice were rendered unconscious by carbon dioxide (CO_2) asphyxiation and killed by cervical dislocation. The tissue samples were placed on ice in ice cold transport medium using Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich Company Ltd., Gillingham, Dorset, UK) without Foetal Bovine Serum (FBS) (Gibco, Life Technologies Corporation, Paisley, Renfrewshire, UK) supplemented, containing 25mM HEPES, with glutamine (Gibco), 0.1mg/ml penicillin-streptomycin (Lonza Ltd, Slough, Berkshire, UK), and 0.3µg/ml Fungizone (Lonza).

In the original protocol at QMUL for establishing osteoblast primary cell cultures, the bones were taken from mice calvaria and ulna. They were cleaned and cut in to small pieces using sterile sharp blade. Then the bone pieces were washed in sterile PBS. The collagenase solution was prepared (Hughes and Aubin, 1998) (Table 2-1). The bone pieces were digested using serial collagenase digestion method (Figure 2-1) as follows: first and second digestion for 10 minutes, third, fourth and fifth digestion for 20 minutes. For each round of digestion, the collagenase solution including cells was collected and put in centrifuged tube containing 100% FBS to stop collagenase action. The solution was centrifuged at 1,100 rpm at 4^{o}C for 5 minutes. The cells were re-suspended in culture medium and grown in T-25 flasks in CO_2 incubator.

Collagenase solution (Hughes and Aubin, 1998) was prepared as follows:

		For 1 mouse (mg)	For 5 mice (mg)
Collagenase	1.5 mg/ml	100	300
DNAse	9.7 units/ml	0.25	0.75 (1,920 units)
Chondroitin sulphate	0.12 mM	79	237
Sorbital	100 mM	1,200	3,600
KCl	111.2 mM	500	1,500
$MgCl_2$	1.3 mM	17.4	52.2
Glucose	13 mM	170	510
$ZnCl_2$	0.5 mM	4.5	13.5
Tris HCl pH 7.4	50 mM	66	198

Table 2-1: Collagenase solution used for serial collagenase digestion method

In later experiments, once the tissue had been stabilised 1 ml of culture medium warmed to 37°C was gently pipetted into each well. All cell culture plates were incubated in a humidified 5% CO_2 atmosphere at 37°C. After 24 hours the plated wells had a further 1 ml of medium added. Media were replaced and growth of cultures checked every three days. Culture medium for all cells consisted of DMEM containing bicarbonate buffer, supplemented with 10% FBS and glutamine, 0.1 mg/ml penicillin-streptomycin and 0.3 µg/ml Fungizone.

Once the cells in each well reached 80-90% confluence, they were passaged into T-25 flasks (Nunc, Themo Fisher Scientific, Rochester, NY, US). They were washed briefly with 2.5 ml of sterile PBS. Following removal of the PBS, the cells were detached from the surface (trypsinised) using 500 µl of 0.5% Trypsin/0.2% EDTA (Lonza) at 37°C for approximately 2 minutes. After checking microscopically that the cells had detached, 2.5 ml of medium was added to each well and mixed thoroughly by pipetting to disaggregate the cells further. The contents of each well were then placed into individual T-25 flasks. All cultures were subsequently expanded and passaged using a seeding density of 5×10^5 per flask until sufficient cells were available for experiments.

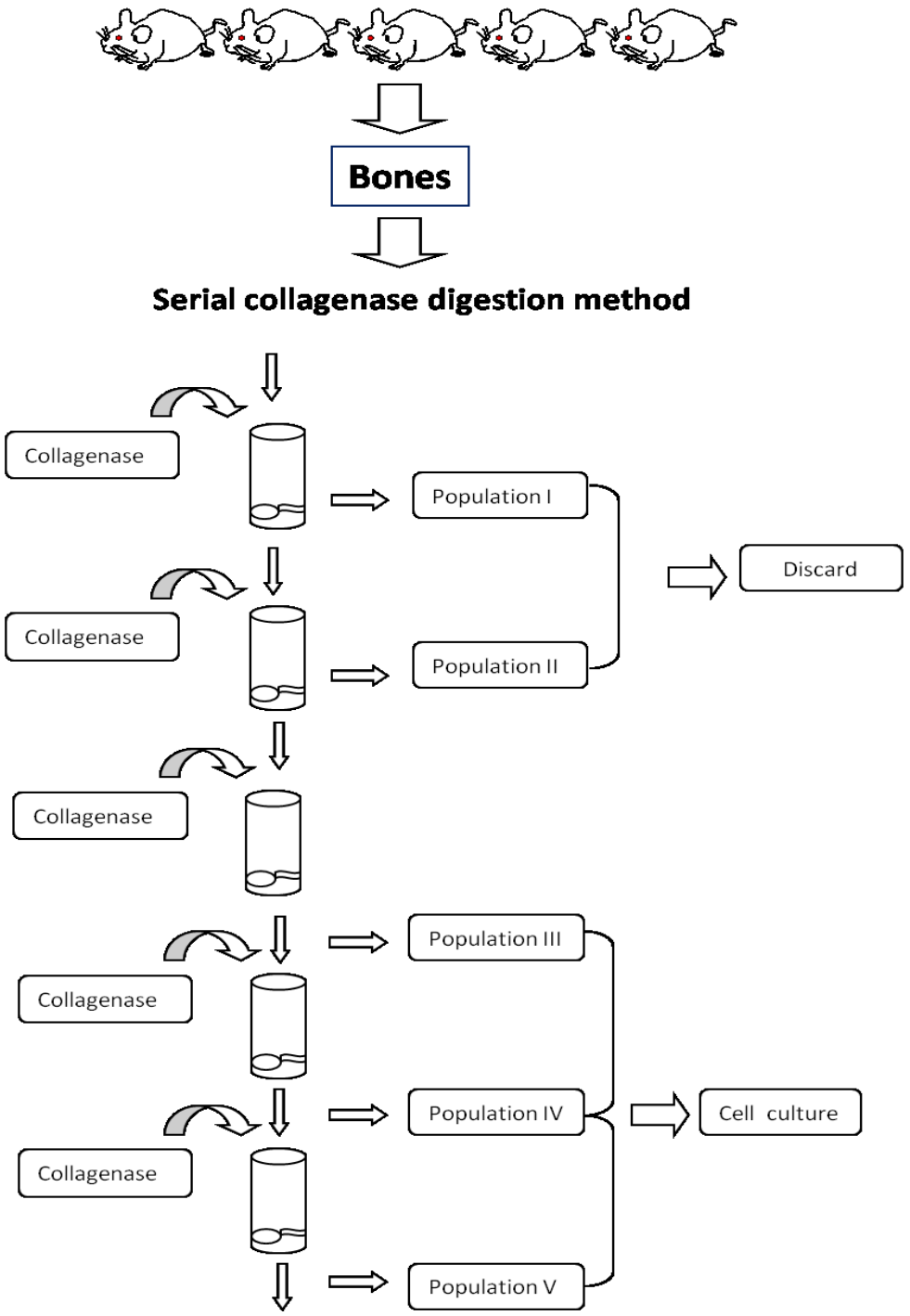


Figure 2-1: The serial collagenase digestion method. The population I and II from the first and second round of collagenase digestion were discarded. The population III, IV and V from the third, fourth and fifth collagenase digestion were collected and grown in culture.

2.2.2 Establishing adult mice osteoblast cells using explantation method

The first group of 5 male mice (weight 22.74g±1.29) were obtained. The bones were taken from calvaria and ulna matched pairs from each mouse using the explantation method (Hughes and Aubin, 1998). At that first passage, osteoblast-like cells from ulna observed better proliferation when compared to osteoblast-like cells from calvaria. Then at passage 2, both osteoblast-like cells from calvaria and ulna showed slow proliferation and underwent senescence. This method was tried on 5 separate occasions but in all cases cells did not survive beyond passage 2. We were able to explant 3 matched pairs of osteoblast-like cells from calvariae and ulnae from 30 mice.

2.2.3 Establishing adult rat primary cell cultures using explantation and collagenase isolation methods

The different methods used in this study to establish dermal fibroblasts, BMSCs, calvarial osteoblasts and femoral osteoblasts are described as follows:

Skin:

Primary dermal fibroblasts were prepared by explant culture (Kobayashi et al., 2008). Four different areas of connective tissues from scalp, tissue overlying shoulder, abdominal and ulna area were taken (about 2×2 cm) from male Wistar rat (210 g, 10 weeks old, London, UK). The hair and superficial part of skin were removed with a sharp scalpel. The tissues samples were cut into 1×1 mm in size and placed onto the surface of sterile 6-well cell culture plates (Nunc). The tissue from different animals was plated separately allowing comparison between cells from different animals. Approximately five pieces were placed in each well. They were left undisturbed 3 days in DMEM with 10% FBS and glutamine, 0.1 mg/ml penicillin-streptomycin and 0.3 μg/ml Fungizone at 37°C in a humidified 5% CO_2 atmosphere. Approximately 1 week later, the explanted rat dermal-derived fibroblasts were harvested by trypsinisation and continued culturing in T-25 flasks (Figure 2-2). The rat dermal-derived fibroblasts were grown up to passage 5 to reach sufficient cell numbers and used in experiments.

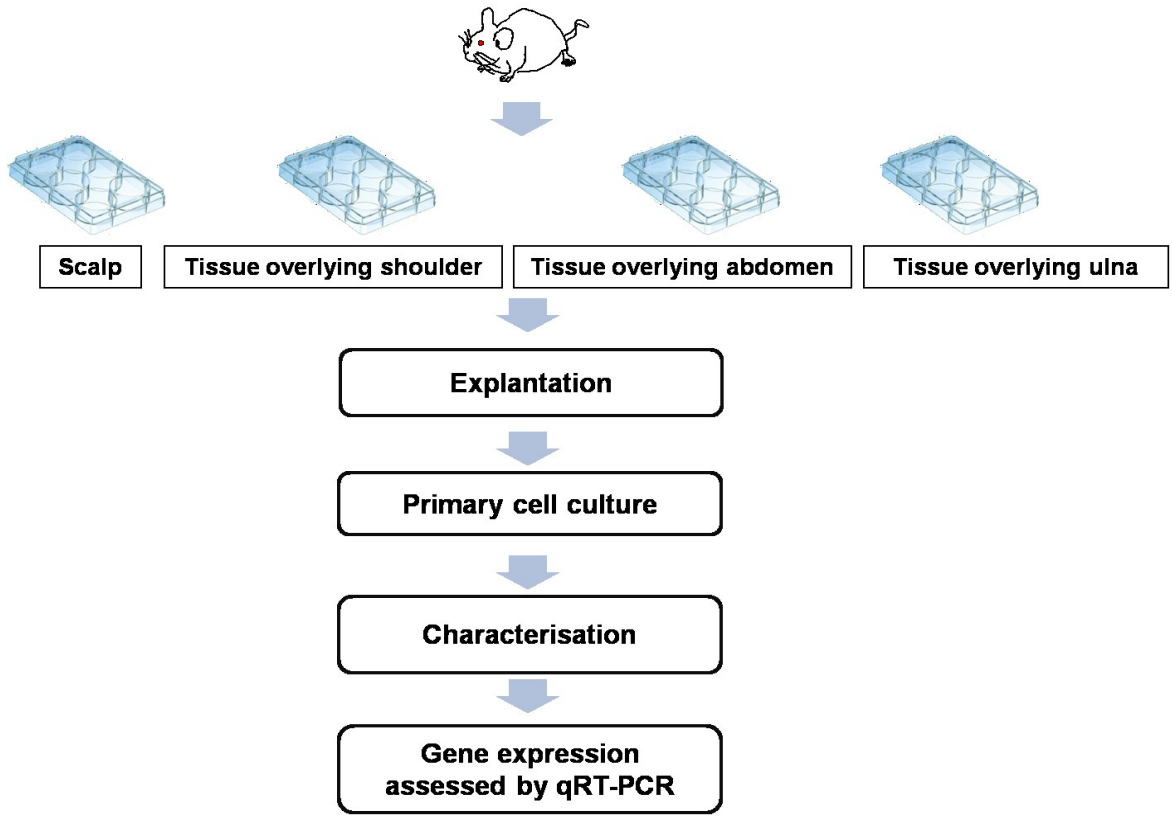


Figure 2-2: Tissues from 4 different sites were taken. The tissues were explanted and grown in fibroblast culture medium, characterised and later tested for *Hoxa* gene expression.

In later experiments tissue from four rats were used. In these experiments, only three different sites from each rat were taken - from the scalp, tissue overlying the abdomen, and tissue overlying the femur region. Tissue overlying the ulna region was not taken, but tissue overlying femur was taken instead as it may provide more related data on osteoblasts derived from femurs (Rinn et al., 2006).

Bone Marrow Stromal Cells (BMSCs):

Initially, BMSCs were cultured, collected and kindly provided by I.J McKay. Briefly, BMSCs were removed from the ulna, femur, tibia, humerus and ribs of five rats (100-110 g), and plated separately into T-25 flasks. After initial plating the non-adherent cells were removed and cells cultured until they were 90% confluent. Cells were then lysed and total RNA extracted. *Hoxa* gene expression was assessed by qRT-PCR (Taqman) to investigate levels of *Hoxa1*, *Hoxa2*, *Hoxa4*, *Hoxa5*, *Hoxa7*, *Hoxa10*, *Hoxa13*, *Alp*, *Bglap*, and *Tgfbi*.

In later experiments, BMSCs were removed from femur from different groups of rats (Rats 1-4, Rats 5-8 and Rats 9-12). Briefly, bony shafts were removed aseptically and transferred to normal culture medium. Attached muscles and soft tissue were removed with care. The ends of the bone were sectioned identically to leave a portion of the mid-shaft. The marrows were taken out by flushing culture medium using 1 ml syringe. The cells were aspirated followed by several flushes through the bone until all the bone marrow was flushed out of the bone. The same procedure was performed from the other end of the bone (Polisetti et al., 2010). The marrow thus obtained was suspended by pipetting the large marrow cores through a 1 ml pipette and plated separately for each rat into T-25 flasks in culture medium. The cultures were maintained at 37^{o}C in a humidified 5% CO_2 incubator and left undisturbed for 48 hours. After 48 hours of initial plating, the non-adherent cells were removed. The media was changed every three to four days. The adherent cells were cultured in normal growth medium. When the cells reached 80-90% confluence, the cultures were passaged and continued to grow. The cultures were harvested with Trypsin-EDTA solution (0.25% trypsin, 1mM EDTA, Sigma).

Calvaria:

The calvariae were aseptically removed from different groups of rats (Rats 1-4, Rats 5-8 and Rats 9-12) immediately after death and transferred to culture medium. The parietal skull bones were then cut with a scalpel to produce two equal sized rectangular portions of contralateral parietal bone free of suture and other attendant soft tissues (Hughes and Aubin, 1998). Bone portions were washed with sterilized phosphate-buffered saline (PBS). Then they were cut into pieces approximately 1 mm^3, put in sterile universal tube and shaken vigorously to remove soft tissue pieces, blood, fat and debris. If the PBS was not clear, then another wash was required. The bone chips were transferred evenly into 6-well culture plates containing DMEM supplemented with 10% foetal bovine serum with glutamine and 1% P/S and incubated at 37^{o}C in 5% CO_2 incubator, left undisturbed for 48-72 hours until an 'osteoid seam' was noticed. This usually occurs 3-5 days after the culture was set up. When plentiful adherent cells of seam formation were observed, the bone chips were collected and followed by trypsin/collagenase digest procedure (Tsiridis et al., 2006). Briefly, medium from dishes of bone chips were removed. The bone chips were carefully transferred to a 20

ml sterile universal tube. The trypsin/collagenase was added to the chips, incubated on Rolamixer for 20 minutes.

The trypsin/collagenase for osteoblast isolation was prepared as follows:

Collagenase powder (C9891-100 mg)	100 units/ml
Trypsin (Gibco 15400-054)	0.02%
Hepes 1 M (H0887) (Final concentration 0.01 M)	1 ml
PBS	100 ml

The cloudy fluid in the universal tube was observed which indicated that the cells had been in the solution. The supernatant was carefully transferred to a new universal tube and centrifuged at 2,000 rpm at 18^{o}C for 5 minutes. The chips were rinsed with culture medium and placed in 60 mm dishes with fresh culture medium for further growing.

The cell pellet was observed at the bottom of the universal tube after centrifugation. The medium was removed. The cell pellet was re-suspended, centrifuged at 2,000 rpm for 5 minutes and washed 2 times with culture medium. The cells were further grown in culture medium supplemented with 10 ng/ml of FGF-2 in 6-well culture plate (Nunc) and incubated at 37^{o}C in 5% CO_2 incubator as summarised in Figure 2-3.

Femur:

The femur shafts were prepared as described (Hughes and Aubin, 1998; Tsiridis et al., 2006). Briefly, rats were put to sleep by Nitrous Oxide and killed by cervical dislocation. Left and right femurs were removed aseptically and transferred to culture medium. Attached muscles and soft tissue were removed with care to leave the periosteum intact. The ends of the bone were sectioned identically to leave a 12 mm portion of the mid-shaft. The marrows were taken out by flushing with syringe. The bones were cut longitudinally into 2 pieces, and then cut into pieces approximately 1 mm^3 and transferred into 6-well culture plate in culture medium then incubated at 37^{o}C in 5% CO_2 incubator and left undisturbed for 48-72 hours until an 'osteoid seam' was noticed. When plentiful adherent cells of seam formation were observed, the bone chips were collected and followed by trypsin/collagenase digest procedure and grown as previously described for further experiments.

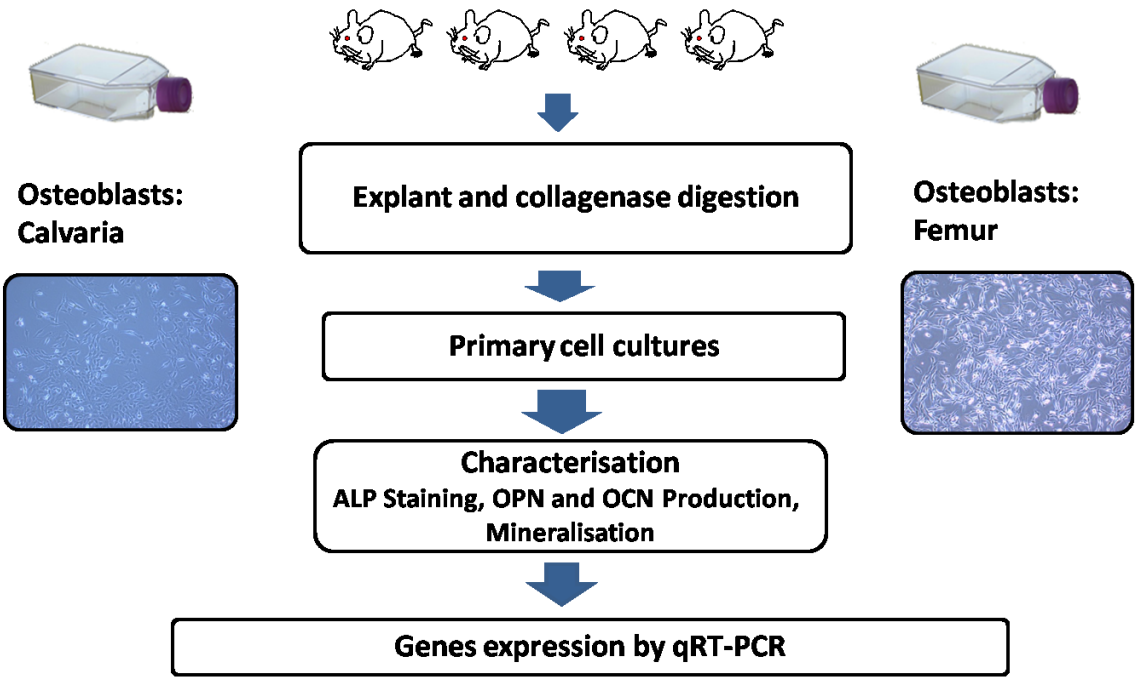


Figure 2-3: Bones from calvaria and femur were taken from 4 rats, matched pairs. The bones were cut into small pieces, explanted and grown in osteoblast culture medium supplemented with FGF-2. They were characterised and tested for their osteoblast-associated gene expression such as *Runx2*, *Bglap* and *Spp1*. In addition, *Hoxa* gene expression and transcription factors such as *Msx2*, *Irx5* and *Tbx3* were also tested.

2.3 Cell doubling

To test the effects of FGF-2 on further cell growth, at p5 cultures, were continued either remaining in medium including FGF-2, or without FGF-2 supplementation. Cells continued to grow and remained viable up to p9 at least, in both sets of cultures.

Briefly, $5x10^5$ cells from each of 4 osteoblastic cells at p5 were seeded in T-75 flasks and grown separately; one group grown in osteoblast culture medium without FGF-2 supplementation and other group grown in osteoblast culture medium supplemented with FGF-2.

The cells from two groups were count using cell counter every 5 days. Normally, the cells that have been grown in osteoblast medium supplemented with FGF-2 become confluent in 5 days, whilst the cells grown in osteoblast medium without FGF-2

supplementation have not reached confluent in 5 days. They were counted every 5 days from p5 to p9.

The overall number of cell doublings was calculated. Data were calculated for population doublings (PDs) = the log of the ratio of the final count (N) to the starting (baseline) count (Xo), divided by the log of 2 (Greenwood et al., 2004a, b); that is

$$PD = [\log(N \div Xo)] \div \log 2.$$

2.4 Non-Radioactive Cell Proliferation Assay

The CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega, Southamton, Hampshire, UK) is a colorimetric method for determining the number of viable cells in proliferation or chemosensitivity assays. It is composed of solutions of a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine methosulfate; PMS).

The mitochondria in viable cells reduce MTS into a water-soluble coloured formazan product in tissue culture medium, the intensity of which is quantified by reading the absorbance of the formazan at 490 nm using a spectrophotometer (Dynex Technologies reader). The MTS solution and the PMS solution were completely thawed approximately 90 minutes at room temperature. The entire content of the 1 ml tube of PMS solution was transferred to the 20 ml bottle of MTS solution. This mixture was aliquoted in foil-wrapped bijou containing 2 ml of the mixture and stored at -20^{o}C between uses.

The 96-well plates of treated experiments were taken from the incubator. 20 µl of the combined MTS/PMS solution was pipetted into each well containing 100 µl in culture medium. The plates were then incubated at 37°C in a humidified, 5% CO_2 atmosphere for 1 hour. The plates were read at 490 nm using a spectrophotometer. To eliminate the products from the MTS assay reaction, all wells were washed three times with PBS prior to determining total alkaline phosphatase activity.

2.5 Determination of total alkaline phosphatase activity

The osteoblastic activity of cells was determined by measuring alkaline phosphatase (ALP) activity using *p*-nitrophenyl phosphate. The ALP activity was determined by measuring the conversion of the colourless substrate *p*-nitrophenyl phosphate into the yellow coloured reaction product *p*-nitrophenol (PNP) and inorganic phosphate by the ALP enzyme. Briefly, the ALP cleaves the phosphate group to reaction product, *p*-nitrophenol which is yellow at alkaline pH.

The ALP substrate solution was freshly prepared per 96-well plate to be assayed: 8.2 ml distilled water, 2.4 ml 1.5 M alkaline reaction buffer (Sigma-Aldrich Company Ltd, Gillingham, Dorset, UK), 30 mg N-Nitrophenylphosphate disodium salt hexahydrate (Sigma-Aldrich), and 1.2 mM $MgCl_2$.

100 µl of substrate solution was pipetted into each well using a multichannel pipette. The plates were incubated at 37°C for 20 minutes (Hughes and Aubin, 1998) and the reaction stopped by pipetting 100 µl 0.5 M NaOH into each well. Once the reaction had been stopped, the reaction product was measured using spectrophotometer at the wavelength of 405 nm.

2.6 ALP Histological staining

Medium was removed from 6-well plates and cells rinsed in cold PBS. The cells were fixed in 10% cold neutral formalin buffer for 15 minutes on ice. Buffer was removed and plates were rinsed once with distilled water and left in distilled water for 15 minutes. In the mean time, the substrate was freshly prepared, consisting of:

a) Naphthol AS MX-PO_4	5 mg	1 mg (Sigma N4875)
b) DMF (N,N-Dimethylformamide)	200 µl	40 µl (Sigma D4551)
c) 0.2 M Tris-HCL pH 8.3	25 ml	5 ml
d) Distilled water	25 ml	5 ml
e) Fast Red Violet LB Salt	30 mg	6 mg (Sigma F3381)

Distilled water was removed. Fast red salt was added to solution a, b, c, d and filtered with Whatman no 1 filter paper into a container just before staining. The plate was incubated for 45 minutes at room temperature. The plate was rinsed with distilled water 3 times and left in distilled water for 1 hour.

Preparation for 10% Neutral formalin buffer:

a) Formalin/Formaldehyde	100 ml	10 ml
b) Na_2HPO_4	16 gm	1.6 gm
c) $NaH_2PO_4.H_2O$	4 gm	0.4 gm
d) Distilled water to	1 Litre	100 ml

2.7 Osteopontin (OPN) ELISA

Osteopontin (rodent), EIA Kit (Enzo Life Sciences (UK) LTD, Matford Court, Exeter, UK) (Catalog No: ADI-900-090A) was used in this experiment that contained the following reagents:

1. Assay Buffer (Tris buffered saline containing BSA and detergent)
2. Osteopontin (rodent) Standard (lyophilized recombinant rodent osteopontin) that was reconstituted to concentrations of: 100, 50, 25, 12.5, 6.25 and 3.13 ng/ml
3. Osteopontin (rodent) Clear Microtiter 96-well Plate coated with a monoclonal antibody specific for osteopontin
4. Osteopontin (rodent) EIA Antibody, a polyclonal antibody specific for osteopontin
5. Osteopontin (rodent) EIA Conjugate (streptavidin conjugated to horseradish peroxidase)
6. Wash Buffer Concentrate (Tris buffered saline containing detergents)
7. Tetramethylbenzidine (TMB) Substrate (3,3´ 5,5´ -tetramethylbenzidine) and hydrogen peroxide
8. Stop solution (1N solution of hydrochloric acid in water)

Six standards in the range of 3.13-100 ng/ml were prepared. Samples were diluted at 1:16 as recommended by the assay manufacturer. Sample buffer (blank), standards, and samples (50 µl/well) were pipetted in the wells in duplicates. The plate was sealed and incubated for 1 hour shaking (speed range from 120-700 rpm) at room temperature.

After a thorough wash 4 times with wash buffer (400 µl/well), the plate was aspirated and firmly tapped on a lint free paper towel to remove any remaining wash buffer. The secondary antibody (50 µl/well) was added to each well except the blank. After that, the plate was sealed and incubated for 1 hour shaking at room temperature. After a thorough wash 4 times as above, the conjugate (50 µl/well) was added to each well except the blank. The plate was sealed and incubated for 30 minutes at room temperature with continuous shaking.

After the final wash, the substrate solution was added to all wells and incubated for 30 minutes at room temperature with continuous shaking. Then the stop solution (50 µl/well) was added to each well and absorbance measured at 450 nm.

2.8 Osteocalcin (OCN) ELISA

The concentrations of osteocalcin in rat osteoblast conditioned media range from <1 ng/ml to 400 ng/ml. The range depends on the cell type and culture condition. Washing and growing cells in serum free media for 24-48 hours were recommended prior to measuring osteocalcin levels as Bovine IgG and osteocalcin, the bovine serum products, can interfere with the assay.

The samples were prepared by using cells lysing method. The cells were grown in osteoblast differentiation medium for 14 days. The medium was removed. After that, 1 ml of distilled water was added and frozen for 15 minutes at -80^{o}C. Then the samples were thawed for 20 minutes at 37^{o}C. The samples were resuspended with a sterile pipette. The freeze-thaw procedure was repeated twice more and the cell lysate was frozen at -80^{o}C until used.

Rat Osteocalcin EIA Kit (Biomedical Technologies, Inc., Stoughton, MA, US) (Catalog No: BT-490) was used in this assay that contains the following reagents:

1. Working sample buffer
2. Phosphate-Saline Concentrate (wash buffer)

3. Osteocalcin Standard 200 ng lyophilized was reconstituted with 2.0 ml of sample buffer to make working standards concentrations of 50, 25, 12.5, 6.25, 3.12, 1.5, 0.78 and 0 ng/ml.
4. Osteocalcin Antiserum Concentrate
5. Donkey anti-Goat IgG Peroxidase
6. Tetramethylbenzidine (TMB) Substrate (3,3´ 5,5´ -tetramethylbenzidine) and hydrogen peroxide
7. Stop Solution
8. Rat Serum Control
9. One 96 well plate coated with osteocalcin antibody

The experiment was performed as a same day procedure according to the manufacturer's instructions as follows:

Seven standards in the range of 0.78-50 ng/ml were prepared. Sample buffer (blank), standards, controls and samples (25 µl/well) were pipetted in the wells in duplicates followed by 100 µl of osteocalcin antiserum in each well; the plate was sealed and incubated for 2.5 hours at 37°C. The wells were aspirated completely and washed five times (by hand) with Phosphate-Saline wash buffer (300 µl/well). Wash buffer was completely removed. Diluted Donkey anti-Goat IgG Peroxidase (100 µl/well) was added to each well; the plate was sealed and incubated for 1 hour at room temperature. The wells were aspirated completely and washed five times with Phosphate-Saline wash buffer (300 µl/well). Mixed solution (1:1 ratio) of TMB solution and Hydrogen Peroxide solution (100 µl/well) was immediately added and incubated in the dark at room temperature for 30 minutes. The stop solution (100 µl/well) was added to all wells and measured absorbance at 450 nm within 15 minutes.

2.9 Mineralised bone nodules with Alizarin red

Alizarin Red S (Sigma-Aldrich) (Sigma A5533) was prepared by adding 1 g of Alizarin red to 90 ml of distilled water and stirred with a magnetic stirrer until dissolved, the pH adjusted to pH 4.2 by adding 0.5M of KOH. After that the solution was transferred to a measuring cylinder and made up to a total volume of 100 ml with distilled water. The solution was filtered through coarse Fisherbrand Filter paper QT 260 (Fisher Scientific Ltd, Loughborough, Leicestershire, UK) before using.

For a 6 well-plate, the medium was removed. The cells were washed with PBS 1-2 ml per well. The PBS was removed and cells were fixed in 10% formal saline for 15 minutes using 1-1.5 ml per well. After that, formal saline was removed and the plate was washed with distilled water using 2 ml per well. The cells were then stained in 1% Alizarin red for 5 minutes using 1 ml per well. The excess stain was removed and washed 5 times with 50% Ethanol approximately 2 ml per well and the last wash was left for 15 minutes. The plate was left to air dry in room temperature.

2.10 CellTracker™ Probes for long-term tracing of living cells

CellTracker™ fluorescent probes (Invitrogen, Life Technologies Corporation, Paisley, UK) are not transferred to adjacent cells in a population and retained in living cells through several generations by the daughter cells inherited the dyes after cell fusion. These reagents pass freely through cell membranes, but once inside the cell, are transformed into cell-impermeable reaction products which claim to have excellent retention, strong fluorescence and relatively uniform cytoplasmic staining.

2.10.1 Preparing dye working solution

The lyophilized product was recommended to dissolve in DMSO to a final concentration of 10 mM. The stock solution was diluted to a final working concentration of 0.5-25 μM in serum-free medium, warmed the working solution to 37^{o}C before using. According to the manufacturers, for long-term staining (more than about 3 days) or the use of rapidly dividing cells 5-25 μM dye is required. Less dye, 0.5-5 μM is needed for shorter experiments.

2.10.2 Staining protocol

When the cells were approximately 80% confluent, the medium was removed from the flask and the pre-warmed CellTracker™ dye working solution was added and incubated for 30 minutes in CO_2 incubator. The dye working solution was replaced with fresh, pre-warmed medium and the cells incubated for another 30 minutes at 37^{o}C. The cells were washed with PBS. Then the cells were grown in culture medium. CellTracker™ Green (C7025 Invitrogen), CellTracker™ Red (C34552 Invitrogen) and CellTracker™ Orange (C34551 Invitrogen) were used in this study.

2.10.3 The maintenance of fibroblast positional identity in heterotypic culture

Fibroblasts were cultured in T-25 flask until they were at least 90% confluent. The cells were then trypsinised and seeded in Falcon 12-well culture plates (Beckton Dickinson, Oxford, Oxfordshire, UK) at a density of 5×10^5 cells per well in 10% FBS DMEM with 1% P/S and glutamine. After 24 hours, cells were stained with either CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate) or CellTracker™ Red CMTPX.

Briefly, the culture medium was removed and pre-warmed CellTracker™ dye working solution was added. During this time, the chloromethyl group of the dye underwent modification or secreted from the cell. Working concentration of fluorescent probes was optimised. Various concentrations (0, 0.5, 1, 2, 5 μM) of cell trackers were tested. 2 μM was selected as an appropriate concentration for further used in fibroblasts staining experiments.

The cells were incubated with dyes for 30 minutes at 37°C in 5% CO_2 incubator, washed 3 times with PBS to remove access dyes before replacing with fresh medium for another 30 minutes up to overnight at 37°C in 5% CO_2 incubator before trypsinisation and mixing them in heterotypic culture. The cells were visualised using an Olympus AX70 fluorescence microscope at 4, 10, 20x magnification. Photos were taken. The cells were maintained in monotypic or heterotypic culture up to 1 week and observed under fluorescence microscope.

2.10.4 The maintenance of osteoblast positional identity in heterotypic culture

This experiment was to test how long the cells can maintain their identity during heterotypic culturing. The calvarial and femoral osteoblastic cells were cultured in T-25 flask until reached 80-90% confluence. The cells were then trypsinised and seeded in Falcon 12-well culture plates (Beckton Dickinson) at a density of 5×10^5 cells per well in 10% FBS DMEM with 1% P/S and glutamine. After 24 hours, cells were stained with either CellTracker™ Green CMFDA or CellTracker™ Red CMTPX or CellTracker™ Orange CMRA.

Briefly, the culture medium was removed and pre-warmed CellTracker™ dye working solution was added. For long-term staining of more than 3 days, it was recommended to use 5-25 μM dye, whereas less dye 0.5-5 μM is needed for shorter experiments. The working concentration of fluorescent probes was optimised. Various concentrations (0,

0.5, 10, 25, 50 μM) of cell trackers were tested. The working concentration of 25 μM was selected as an appropriate concentration for further used in experiments.

The cells were incubated with dyes for 30 minutes at 37°C in 5% CO_2 incubator, washed 3 times with PBS to remove access dyes before replacing with fresh medium for another 30 minutes up to overnight at 37°C in 5% CO_2 incubator before trypsinisation and mixing them in heterotypic culture. The cells were visualised using an Olympus AX70 fluorescence microscope at 4, 10, 20x magnification. Photos were taken. The cells were maintained in monotypic culture or heterotypic culture up to 1 week and observed under fluorescence microscope. However, the cells which stained with CellTracker™ Green had started to decrease their fluorescence intensity within 1 week compared to the cells which stained with CellTracker™ Red. The photos taken at Day 7 in culture of the cells that stained with CellTracker™ Green had become problematic. Despite the fact that the cells were losing their labeling over time in culture and difficult to observe using fluorescence microscope, the cells were later analysed by using a more sensitive method, flow cytometry.

2.11 Flow cytometry

Fluorescence Activated Cell Sorters (FACS machines) make physical separation of sub-populations of cells from a heterogeneous population, with a high degree of purity and high speed possible. This technique can be used in many situations (Cram, 2002; Huh et al., 2005; Nunez, 2001; Rieseberg et al., 2001). Various methods can be used in flow cytometric cell sorting:

1) Mechanical sorting

The mechanical device called a catcher tube can be used to sort the cells by moving into the stream to collect the cells when cells of interest passing through the laser beam. The system can determine if each cell belongs to the selected population defined by their boundaries in the cytogram. The catcher tube will capture the cell of interest and collect into a tube otherwise it will be dispatched to the waste tank. This is a very slow method for sorting and is now rarely used. It is however good for very large cells or known biohazardous cells, as the whole system is enclosed and no aerosols are generated.

2) Electrostatic sorting

By applying a vibration to the nozzle, the particles are forced to disintegrate into a stream of droplets. The system can detect the droplets with particles of interest from the stream by applying a charge to those droplets. When a charged droplet passes through an electrostatic field, it is redirected and collected into the corresponding collection tube. The droplet is deflected in the direction of the opposite charged plate, so that this droplet is separated from uncharged and oppositely charged droplets.

3. Other methods

Another way to sort cells is to use magnetic beads. It is possible to confidently select cells of interest by adding magnetically labeled antibodies to the study population or by negatively selecting a population using coupled antibodies to magnetic beads specific for the cells other than those of interest. Cells are then passed between a magnetic field through a column to either elute or impede the population of interest.

2.11.1 Flow Cytometry for analysis

The BD FACSCanto II was used for flow cytometry analysis. Briefly, the cells were stained at Day 0 which gave the strongest fluorescence intensity and used as a positive cell staining, whilst the unstained cells which gave the negative fluorescence were used as a negative control. The cells stained with DAPI also been used to identify dead cells in our early experiments. We found that our cells were healthy in general. Therefore, DAPI were not need to be added in later experiments. These cells were used to calibrate the FACS machine and gate unwanted cells before starting the analysis.

To test for dye contamination, the experiments were set up using monotypic or heterotypic cultured cells which labelled with CellTracker^{TM} dyes. They were counted and each cell type of $1x10^5$ cells were grown in T-25 flasks in monotypic or heterotypic cultures for 0, 3 and 7 days. Then the cells were detached from tissue culture flasks with Trypsin/EDTA and collected into sterile FACS tubes. The cell pellet was centrifuged for 5 minutes at 1,100 rpm. The cells were re-suspended in 500 µl normal culture medium and ready to be analysed. Standard flow cytometry was used for determining the percentage of cells in the population that are in S-phase. BD FACSDiva been used for analysis.

The spectral characteristics of the fluorescent CellTracker™ probes are summarised in Table 2-2.

Cat.no.	CellTracker™ Probe	Abs* (nm)	Em* (nm)
C7025	CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate)	492	517
C34552	CellTracker™ Red CMTPX	577	602
C34551	CellTracker™ Orange CMRA	548	576

Table 2-2: Summary of the fluorescent CellTracker™ probes used in this study. *Absorption and fluorescence emission, determined in aqueous buffer or methanol; values may be different to some extent in cellular environments. CMFDA is colourless and non-fluorescent until the acetate groups are cleaved by intracellular esterases; hydrolysis of the acetates yields a product with the indicated spectral properties.

CellTracker™ Green was the FITC channel at 488 nm excitation, 530/30 collection. CellTracker™ Red was the PE channel at 488 nm excitation, 585/42 collection. CellTracker™ Orange was the PE channel at 488 nm excitation, 585/42 collection.

2.11.2 Flow cytometry for cell sorting

The BD FACSAria II was used for cell sorting according to different cell fluorescence labelling. Briefly, the cells were stained at Day 0 which gave the strongest fluorescence intensity were used as a positive cell staining and the unstained which gave the negative fluorescence were used as a negative control. The cells stained with DAPI also been used to identify dead cells in our early experiments. We found that our cells were healthy in general and DAPI were not need to be added. Unstained cells were used to set optimal side and forward scatter voltages on the FACS machine and to gate out unwanted cells before cell sorting. The sorter was QC checked before each experiment using BD Cytometer Set up and Tracking Beads and the optimal drop delay was determined using BD Accudrop beads. Cells were sorted on a 100 μm nozzle.

All cell sorting experiments were kindly helped by Dr Susanne Heck, PJ Chana and Helen Graves, Flow Cytometry Lab, BRC Core facility, Guy's & St. Thomas' NHS Foundation Trust. Briefly, the cells were labelled with CellTracker™ dyes. They were counted and each cell type of $5x10^5$ cells were grown in T-25 flasks in monotypic or heterotypic cultures for 0, 3 and 7 days. Then the cells were detached from tissue culture flasks with Trypsin/EDTA and collected into sterile FACs tubes and pelleted by

centrifugation for 5 minutes at 1,100 rpm. The cells were re-suspended in 500 µl normal culture medium and kept on ice throughout sorting process. Standard flow cytometry Electrostatic Sorting was used for cell sorting into designated containers. BD FACSDiva software been used for analysis. CellTracker™ Green was the FITC channel at 488 nm excitation, 530/30 collection. CellTracker™ Red was the PE channel at 488 nm excitation, 575/26 collection. CellTracker™ Orange was the PE channel at 488 nm excitation, 575/26 collection.

2.12 RNA extraction

Total RNA was extracted from cells using RNeasy® Mini Kit (Qiagen, Crawley, West Sussex, UK), in accordance with manufacturer's instructions. Briefly, cells were grown in a monolayer up to 80-90% confluent and disrupted by adding 350 µl of buffer RLT which contains 10 µl of β-mercaptoethanol (β-ME) in 1 ml of buffer RLT in the pelletted cells of less than $5x10^6$ cells. The lysate was homogenized using QIAshredder™ spin column (Qiagen). One volume of 70% ethanol was added to homogenized lysate and transferred to RNeasy® spin column. For genomic DNA contamination, 10 µl of DNase 1 stock solution in 70 µl of buffer RDD was added to RNeasy® spin column membrane for 15 minutes at room temperature. The RNeasy® spin column was washed twice with buffer RPE. Then RNeasy® spin column was placed in a new 1.5 ml collection tube and 20-30 µl of RNase-free water directly was added to spin column membrane, centrifuged for 1 minute at ≥10,000 rpm to elute RNA.

2.13 Determination of quantity and purity of RNA

RNA quantification was carried out immediately prior to cDNA synthesis. RNA quantification and purity was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). The spectrophotometer lens was wiped clean with lint-free tissue and subsequently added 1 µl RNase-free water for initialisation. The RNA stored at -80°C was thawed on ice. Once thawed, 1 µl of each sample was transferred onto the pedestal, and the purity was assessed and concentration recorded.

The purity was estimated using the reading ratio at 260 nm and 280 nm (A260/A280). All samples tested were considered acceptable for cDNA synthesis as A260/A280 ratios

were between 1.8 and 2.2. The DNase was treated as part of RNA extraction, gel analysis was not carried out to visually detect genomic DNA contamination.

2.14 cDNA synthesis

1 µg RNA of sample was used for cDNA synthesis. Each RNA sample was diluted in RNase-free water for 1 µg RNA in 14 µl volume. RNA samples were also used to make a control mixture by replacing reverse transcriptase with RNase-free water to make No-RT control. This control was included to check any presence of contaminating genomic DNA in the q-PCR reaction. The diluted RNA samples were pipetted into 0.5 ml PCR tubes containing 1 µl of 0.5 µg/µl Oligo(dT) 15 primer (Promega). The tubes were heated for 5 minutes on a block pre-set at 70°C before used to denature the RNA secondary structure, cooled on ice for annealing of primers to the RNA, and centrifuged briefly to assemble all contents to the bottoms of the tubes.

To each tube, the following were added in order:

3.75 µl RNase-free water
5 µl M-MLV Reverse Transcriptase Reaction Buffer (Promega)
1.25 µl PCR Nucleotide Mix containing 10 mM of each dNTP (Promega)
1 µl M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant (Promega)
For No-RT control, the enzyme was replaced with 1 µl RNase-free water.

The samples were then placed in a PCR machine programmed to heat the tubes to 42°C for 30 minutes for extension, then 85°C for 5 minutes to inactivate the enzyme, then cooling down to 4^{o}C for 5-10 minutes. The samples were then immediately stored at -20°C until needed for the real-time PCR reactions.

2.15 Quantitative real-time PCR (qRT-PCR)

Gene expression in cells was quantified using qRT-PCR against the House Keeping Genes (HKGs) to compare the gene expression among different genes of interest before and after co-culturing. The experiment demonstrated whether or not the cells with different gene expression can induce or be induced by other cells or still maintain their identity. In all qRT-PCR experiments (Table 2-3) throughout this thesis a minimum of 4 replicate samples were used for all tests in MicroAmp® Optical 384-well reaction plate for qRT-PCR.

	Experiment (numbers)	Experiment repeated (times)
HKGs Screening: FBTs for all samples 1 rat, 4 sites	1	0
HKGs Screening: BMSCs for all samples 5 rats, 5 sites	1	0
HKGs Screening: OBTs for all samples 4 rats, 2 sites	1	0
Hoxa genes: FBTs at p-5 in 1 rat, 4 sites	1	0
Hoxa genes: FBTs at p-5 in 4 rats, 3 sites	1	0
Hoxa genes: BMSCs at p-3 in 5 rats, 5 sites	1	0
Hoxa genes: OBTs at p-5 in 4 rats, 2 sites	1	0
Hoxa genes: OBTs at p-10 in 4 rats, 2 sites	1	0
Hoxa genes: FBTs in heterotypic CM in 1 rat, 2 sites, 2 time points	1	0
Hoxa genes: OBTs in monotypic culture in 4 rats, 2 sites, 3 time points	2	2
Hoxa genes: OBTs in heterotypic culture in 3 rats, 2 sites, 3 time points	3	3
Hoxa genes: OBTs in heterotypic culture in 4 rats, 2 sites, 3 time points	2	2
Osteoblast-associated genes in monotypic culture in 4 rats, 2 sites, 3 time points	2	2
Osteoblast-associated genes in heterootypic culture in 4 rats, 2 sites, 3 time points	2	2
Transcription factors in monotypic culture in 4 rats, 2 sites, 3 time points	2	2
Transcription factors in heterootypic culture in 4 rats, 2 sites, 3 time points	2	2

Table 2-3: Table of qRT-PCR experimental set-up

2.15.1 Method of selection of reference genes

Prior to detecting the genes of interest, the most stable reference genes were selected from a panel of 6 using a representative range of experimental cDNA samples from all the different tissue and cell types derived. Using reference genes as internal controls to normalize data and control for variability between samples, plates, instrument and pipetting differences. According to the similarity of their expression profile, two reference genes were selected using a pair-wise comparison to compare geometric mean as a normalisation factor using geNorm software analysis (Vandesompele et al., 2002).

Six HKGs (Table 2-4) were screened in order to select two most stable HKGs to be further used (Vandesompele et al., 2002). Five different primary BMSCs derived from different bony regions were tested. Four different primary fibroblasts derived from different dermal regions were tested afterwards, whereas 8 primary osteoblastic cells matched pairs will be investigated for the genes listed in Table 2-5 and Table 2-6.

Official Gene Symbol	Gene Name	Applied Biosystems Assay ID
Ubc	ubiquitin C	Rn01789812_g1
B2m	β2 microglobulin	Rn00560865_m1
Gapdh	glyceraldehyde-3-phosphate dehydrogenase	Rn99999916_s1
Eif4a2	eukaryotic translation initiation factor 4A2	Rn01404755_gH
Atp5b	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	Rn01756316_g1
RN18S1	RNA, 18S ribosomal 1	Hs99999901_s1

Table 2-4: A list of TaqMan Gene Expression Assays used for qRT-PCR detection of the most stable rat reference genes in samples. Regarding the ABI Assay IDs, "Rn" indicates *Rattus norvegicus,* "Hs" indicates *Homo sapiens*. "_g1" indicates assays that may detect genomic DNA. "_m1" indicates the assays whose probes span an exon junction and will not detect genomic DNA. "_gH" indicates assays designed belong to a gene family with high sequence homology. The assays are designed to give between 10 C_T and 15 C_T difference between the target gene and the gene with the closest sequence homology. An assay, therefore, will detect the target transcript with 1,000 to 30,000-fold greater discrimination (sensitivity) than the closest homologous gene. "_s1" indicates the assay whose primers and probes have been designed within a single exon, and therefore will detect genomic DNA.

Official Gene Symbol	Gene Name	Applied Biosystems Assay ID
Hoxa1	homeo box A1	Rn00565157_m1
Hoxa2	homeo box A2	Rn00561390_m1
Hoxa4	homeo box A4	Rn02088951_s1
Hoxa5	homeo box A5	Rn01763079_m1
Hoxa7 or Hoxa9	homeo box A7 or homeobox A9	Rn01451913_m1
Hoxa10	homeo box A10	Rn01451894_m1
Hoxa13	homeo box A13	Rn01326387_m1

Table 2-5: A list of TaqMan Gene Expression Assays used for qRT-PCR detection of *Hoxa* genes in samples. Regarding the ABI Assay IDs, "Rn" indicates *Rattus norvegicus*. "_m1" indicates the assays whose probes span an exon junction and will not detect genomic DNA. "_s1" indicates the assay whose primers and probes have been designed within a single exon, and therefore will detect genomic DNA.

Official Gene Symbol	Gene Name	Applied Biosystems Assay ID
Msx2	msh homeobox 2	Rn00564331_s1
Irx5	iroquois homeobox 5	Rn01538194_m1
Tbx3	T-box 3	Rn00710902_m1
Runx2	runt-related transcription factor 2	Rn01512298_m1
Bglap	bone gamma-carboxyglutamate (gla) protein	Rn01455285_g1
Spp1	secreted phosphoprotein 1	Rn00563571_m1

Table 2-6: A list of TaqMan Gene Expression Assays used for qRT-PCR detection of transcription factors and osteoblast-associated genes in samples. Regarding the ABI Assay IDs, "Rn" indicates *Rattus norvegicus*. "_m1" indicates the assays whose probes span an exon junction and will not detect genomic DNA. "_g1" indicates assays that may detect genomic DNA. "_s1" indicates the assay whose primers and probes have been designed within a single exon, and therefore will detect genomic DNA.

The first strand of cDNA was synthesized from 1 μg RNA. This was used for amplifications performed with specific primers for *Hoxa* genes (Applied Biosystems, Life Technologies, Cheshire, UK). Primer sequences were designed with the Primer Express® programme from Applied Biosystems. For qRT-PCR analysis in an ABI Prism® 7300 Sequence Detector, the TaqMan® Gene Expression Assays, TaqMan PCR reaction mixtures were set up as suggested by the manufacturer. Briefly, a 5 μl aliquot of cDNA was used in a final volume of 25 μl reaction mixture containing 12.5 μl of 2x Taqman® Universal PCR Master Mix (P/N 4304437), 1.25 μl of Gene mix and 6.25 μl of nuclease-free water (all from Applied Biosystems). Thermal cycler conditions consisted of AmpRease®UNG activation at 50^{o}C for 2 minutes, AmpliTaqGold®DNA polymerase activation at 95^{o}C for 10 minutes and 40 PCR cycles, each of which was

95°C for 15 seconds and 60°C for 60 seconds. All PCR reactions were performed in 4 replicates. Each of the signals was normalized to average of two most stable HKGs in the same reaction. Data were presented as the relative expression to average control genes.

2.16 qRT-PCR set-up

In order to have adequate volume for the PCR reactions, 1,014 µl of ultrapure (18.2MΩ) water was added to the total 26 µl cDNA of each sample and the controls in 1.7 ml PCR tubes to make 40-fold dilutions as recommended from provider. The necessary volumes of primers and probes were then mixed with the appropriate amount of PCR mastermix - ABsolute QPCR ROX Mix (2×) (ABgene Ltd.), to obtain 0.5 µl primers and probes and 5 µl PCR mastermix per well. A separate stock mix for each primer and probe set was made. The mastermix contained thermostable DNA polymerase, reaction buffer, dNTPs and ROX, a passive reference dye for normalisation of the FAM reporter dye signals.

PCR reactions were carried in MicroAmp™ Optical 384-Well Reaction Plates (Applied Biosystems). Pipetting was carried out using a digital pipetter. Each cDNA sample was pipetted at a volume of 5 µl per well. To check for non-specific signal arising from primer dimers or DNA contamination of q-PCR reagents, No template controls (NTCs) where cDNA was substituted with ultrapure water were also pipetted for each gene to be assayed. Subsequently 5.5 µl of the stock mix containing primers, probes and PCR mastermix was added to create a final volume of 10.5 µl per well. All reactions were carried out in singleplex which is only one type of primer pair per well. Each sample was tested in 4 replicates.

When finished pipetting, the plates were sealed with optical adhesive film to reduce the chance of contamination between well and prevent sample from evaporation, centrifuged at 4,000 rpm for 1 minute to gather all contents to bases of the wells.

The PCR reactions were then carried out using the Applied Biosystems 7900HT Fast Real-Time PCR System. The thermal cycling parameters used were an initial hold step of 10 minutes at 95°C to activate the DNA polymerase. This was followed by 40 cycles of 15 seconds at 95°C to denature the DNA followed by 1 minute at 60°C for annealing and extension.

Expression data from Applied Biosystem's SDS2.3 software were retrieved and analysed using Microsoft Excel.

2.17 Analysis of qRT-PCR data

Data were normalised against the selected reference genes. The fractional PCR cycle used for quantification, termed *quantification cycle* (Cq), for the means of the replicates was normalized to the average Cq of the controls per plate by subtracting the average Cq of the controls from each mean to give the Cq which is equivalent to the log2 difference between endogenous control and target gene. A raw Cq of 35 was set as the limit of detection in this study: individual replicates which gave Cq values >35 were considered not detected. Relative expression to average control genes data were generated using comparative different genes within samples ($2^{-\Delta Cq}$) method. Stimulation data were processed using the comparative ($2^{-\Delta\Delta Cq}$) method.

2.18 Assumptions regarding qRT-PCR

Applied Biosystems announce the efficiency for the product amplification using their assays is 100%, i.e. there is a doubling of PCR product at every PCR cycle and also state the $2^{-\Delta\Delta Cq}$ method for relative quantification is better related with expected gene expression fold change than methods using pre-determined amplification efficiency for individual assays.

2.19 EdU incorporation

Previous methods used to measure a cell's ability to proliferate by directly measuring DNA synthesis have been by incorporation of radioactive nucleosides; i.e. H-thymidine or by antibody-based detection of the nucleoside analogue bromo-deoxyuridine (BrdU).

Click-iT® EdU Flow Cytometry Assay Kits (Invitrogen A10202) are new alternatives to the BrdU assay. EdU (5-ethynyl-2′-deoxyuridine) is a nucleoside analogue to thymidine. During active DNA synthesis, EdU is incorporated into DNA. Detection is based on a click reaction, a copper catalyzed covalent reaction between an azide and an alkyne. In this application, the EdU contains alkyne, while azide can be detected in the Alexa Fluor® 488 dye, Alexa Fluor® 647 dye, or Pacific Blue™ dye.

Briefly, the cells were incubated with Click-iT® EdU for 48 hours. They were harvested, fixed and permeabilized for 15 minutes. The detection of Click-iT® EdU was incubated for 30 minutes at room temperature and prevented from light. The cells were analysed by flow cytometry. The BD FACSCanto II was used for flow cytometry analysis. For the detection of EdU with Alexa Fluor® 647 azide, it was recommended to use 633/635 nm excitation with red emission filter (660/20 nm or similar). The Alexa Fluor® 647 azide was the APC channel at 633 nm excitation, 660/20 collection to see proliferating cells that have incorporated EdU providing the percentage of cells in S-phase of DNA synthesis.

2.20 Statistical analysis

Data are expressed as means ± SD. In Chapter 4, One-way ANOVA and Bonferroni's Multiple Comparison test used to compare more than two different sites for each gene expression. Additional analyses in Chapters 4 and 6, Wilcoxon's Sign-Rank test were used to analyse cells derived from each animal separately. Paired *t*-test was used to determine whether differences between two different populations were statistically significant. Statistical analysis was carried out using Prism 5 v5.0 software (GraphPad Software Inc.). $p<0.05$, $p<0.01$ and $p<0.001$ considered statistically significant unless stated otherwise.

Chapter 3

Chapter 3: Establishment of osteoblast cultures

3.1 Introduction

The overall aim of the studies in this thesis is to investigate regional differences in osteoblasts derived from different sites, including programming of their "positional identity" and associated phenotypic variations between cells. In order to carry out these studies, it was necessary to be able to isolate and culture osteoblastic cells from a range of regionally distinct sites, and to maintain them in culture for a sufficient period of time to have enough cells for experiments, whilst maintaining their original phenotypes. Throughout these studies we adopted a "matched pair" strategy, whereby pairs of primary osteoblast cultures from different sites were obtained from the same animal, so that comparison between cells were always controlled for between animal variations. This approach potentially provides a technical challenge, as the number of cells isolated from an individual site in a single animal is often quite low, and therefore it was necessary to determine if it was possible to do this and expand the cells sufficiently by long term culture.

In previous studies in our laboratory, Rawlinson successfully isolated and established rat osteoblast primary cells from distinct bony regions using a collagenase isolation method (Rawlinson et al., 2009b). These cells were subsequently tested by microarray to test for preferentially expressed genes between two distinct osteoblast populations from calvarial (paraxial mesodermal-derived) cells and femoral (lateral plate mesodermal-derived) cells.

Use of mice as a source of cells has the potential benefit of better availability of reagents for further study, and ultimately potentially the availability of transgenic animals. However, against this the initial donor tissues are even smaller than those for rats and thus harvested cells may not be sufficient for carrying out our planned experiments. Failing that, we would need to isolate and establish calvarial and femoral osteoblastic cells from adult rat matched pairs.

3.2 Aims

Therefore the aims of this chapter are:

1. To isolate and establish rodent primary osteoblast cultures from distinct bony regions as matched pairs;

2. To test the capacity to expand these cultures extensively whilst maintaining their osteoblastic phenotype.

3.3 Materials and Methods

3.3.1 Osteoblast cultures from mice

Two different cell isolation methods were tested to establish an adult mouse derived primary osteoblast culture. Firstly, tissues from 5 adult mice were obtained and cells released by collagenase digestion as described in section 2.2.1. In further experiments, tissues were taken and explanted as described in section 2.2.2.

3.3.2 Osteoblast cultures from rats

A combination of explantation and collagenase isolation methods were used to establish adult rat osteoblastic cells from calvariae and femurs, as described in section 2.2.3. The cells isolated by explants and collagenase digestion methods of cell isolation were grown separately in osteoblast culture medium. In later experiments osteoblast culture medium was supplemented with 10 ng/ml of FGF-2.

3.3.3 Maintenance of osteoblastic phenotype with continuous culture

Successfully isolated continuous 8 different matched pairs of primary osteoblast cultures from 4 rats were grown up to passage 10 to check whether the cells could still maintain their osteoblastic phenotype up to this passage. In latter experiments, the cells grown within these passages were used. The cells were tested at passage 5 and passage 10 for persistence of osteoblast phenotype, measuring proliferation (section 2.4), ALP staining (section 2.6), osteopontin and osteocalcin production by ELISA (section 2.7 and 2.8), mineralised bone nodules (section 2.9), and expression of a range of genes associated with the osteoblast phenotype (section 2.12-2.18), as summarised in Figure 3-1.

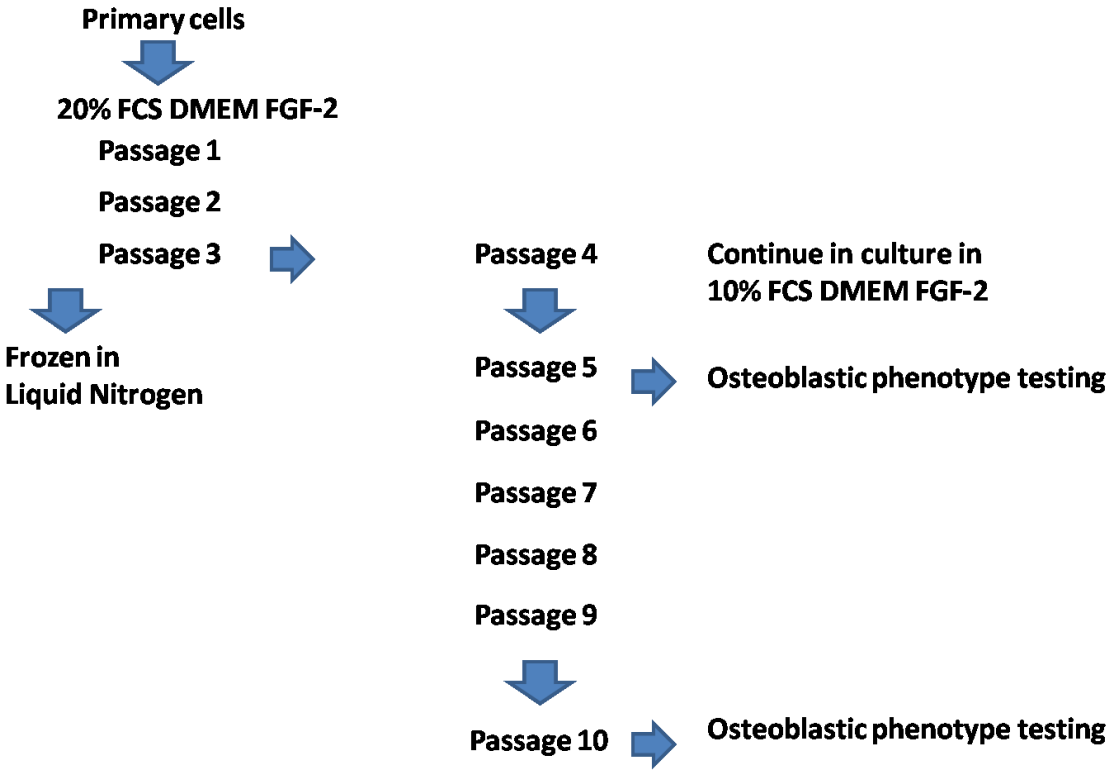


Figure 3-1: This flow chart summarised how adult rat osteoblastic cells were isolated, grown, frozen and tested at different passages.

3.4 Results

3.4.1 Adult mouse osteoblastic cells

In initial experiments, calvarial and ulna bones of 5 adult mice were isolated and treated with collagenase to release osteoblastic cells. A few cells were observed in cultures but failed to grow significantly. In further experiments using explant culture of calvariae and ulnae, cells were initially observed to grow out of the explants from some mice (Figure 3-2) and maintained in culture for periods up to 28 days. Cells derived from calvariae tended to have a round or cuboidal shape, whilst ulna-derived cells tended to appear more spindle shape. However, none of the cultures approached confluence, and later became flattened and apparently senescent as the cells were not dead, but did not proliferate or grow as usual. These cell isolations were repeated up to 5 times, without successfully establishing cultures for further experiments.

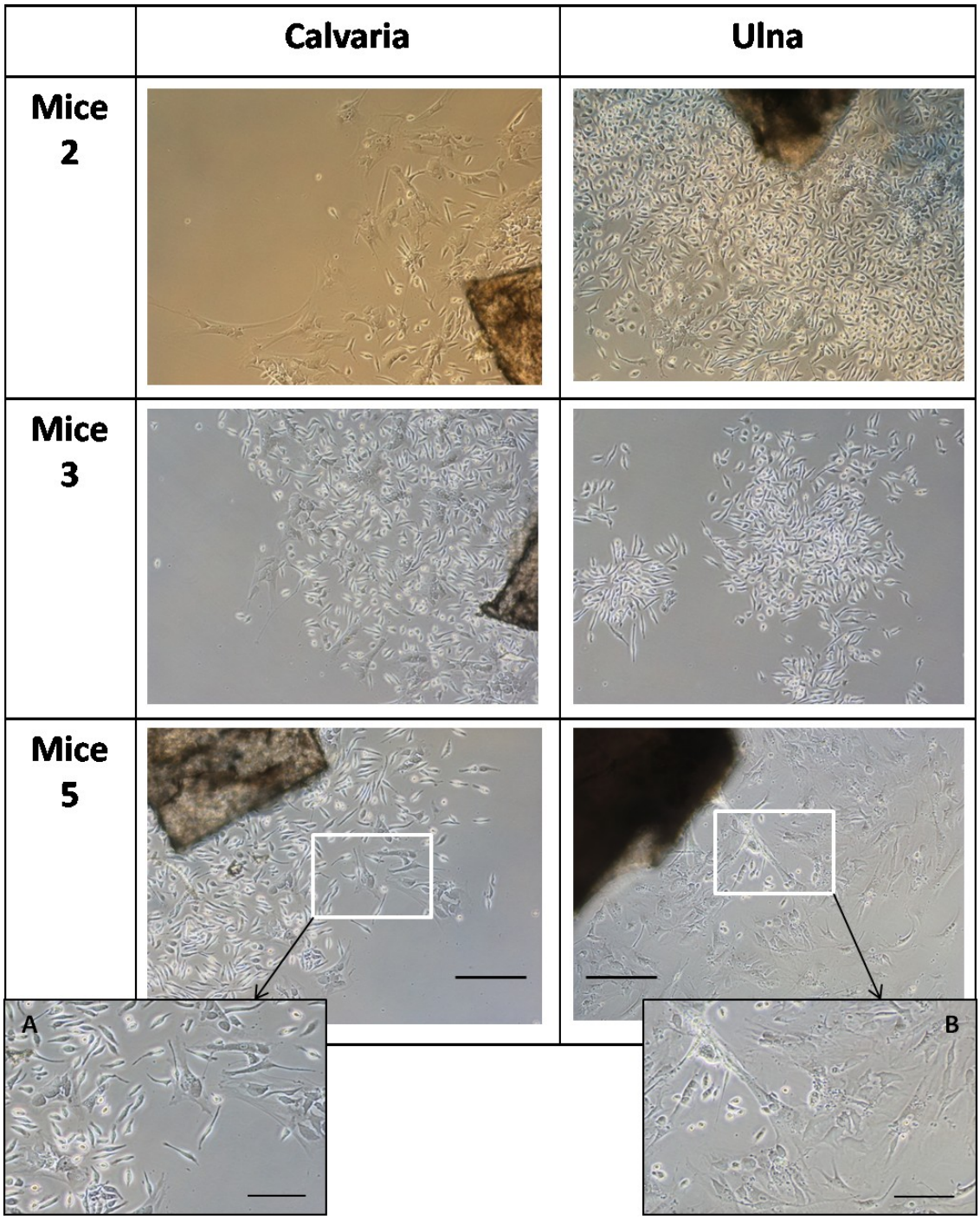


Figure 3-2: Adult mice osteoblastic cells from calvaria and ulna matched pairs from 3 mice in group 2, 3 and 5 were observed using explant isolation method, magnification 100, scale bar = 200 µm. Cells derived from calvaria (A) tended to have a round or cuboidal shape, whilst ulna-derived cells (B) tended to appear more spindle shape, magnification 200, scale bar = 100 µm.

3.4.2 Adult rat osteoblastic cells

For rat osteoblast explant cultures, after about 3 days in culture marked outgrowths of osteoblastic cells were seen from both calvarial and femoral bone explants. With prolonged culture for periods of up to 28 days cultures approached confluence, and were subcultured into T-25 flasks. However cell proliferation tended to cease and cells underwent senescence typically at p1. Occasionally cell cultures were maintained until a second passage into T-25 flasks but then ceased growth. These experiments were repeated twice with 4 animals on each occasion (Figure 3-3).

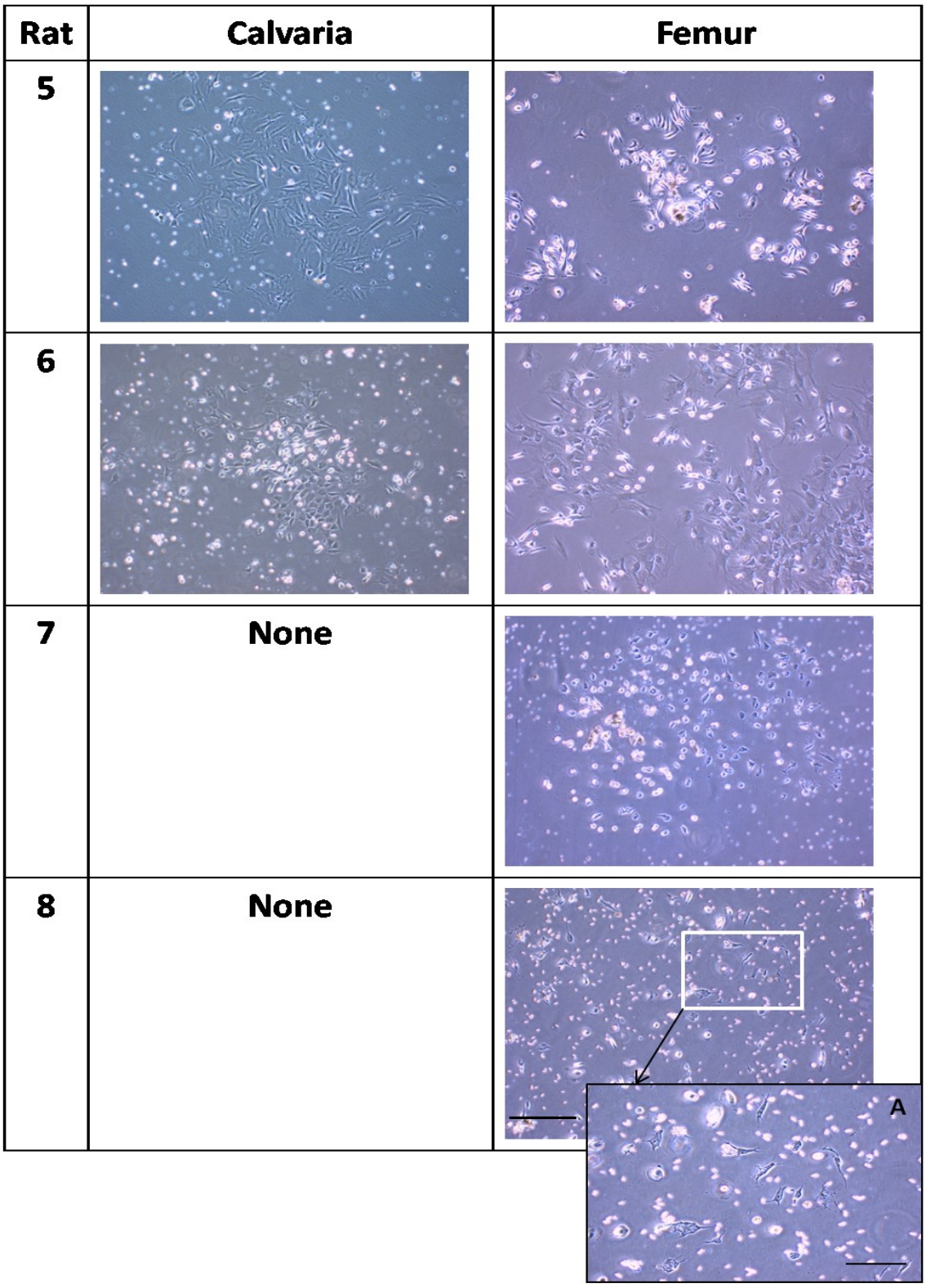


Figure 3-3: After explantation from 4 male rats (Rats 5-8) matched pairs. Occasionally cell cultures were maintained until a second passage into T-25 flasks up to 28 days but then ceased growth, magnification 100, scale bar = 200 µm, (A) at magnification x200, scale bar = 100 µm.

3.4.3 Effect of FGF-2 supplementation

In view of the failure of cultures to undergo sufficient growth *in vitro* prior to apparently reaching senescence, we next tested the effects of supplementing the culture medium with FGF-2 on growth of cultures. In rat osteoblast explant cultures, after about 3 days in culture marked outgrowths of osteoblastic cells were seen from both calvarial and femoral bone explants (Figure 3-4). It was apparent that cells grew more rapidly and could be maintained for multiple passages following the addition of the FGF-2 to the culture media. Thus cells were grown in continuous culture and at passage 3 some of the cells were frozen for future uses. Cells were tested for maintenance of osteoblast phenotype at p5 and again at p10.

To test the effects of FGF-2 on further cell growth at p5, (all of which had been maintained in FGF-medium until that point) cultures were continued in medium either including FGF-2, or without FGF-2 supplementation. Cells continued to grow and remained viable up to p9 at least, in both sets of cultures. However the cell growth was greatly enhanced in cultures with FGF-2. Briefly, $5x10^5$cells from each of 4 osteoblastic cells at p5 were seeded in T-75 flasks and grown separately; one group grown in osteoblast culture medium without FGF-2 supplementation and other group grown in osteoblast culture medium supplemented with FGF-2. The cells from two groups were counted using cell counter every 5 days. Normally, the cells that have been grown in osteoblast medium supplemented with FGF-2 become confluent in 5 days, whilst the cells grown in osteoblast medium without FGF-2 supplementation had not reached confluent in 5 days. They were counted every 5 days from p5 to p9. The overall number of cell doublings was calculated. Data were calculated for population doublings (PDs) = the log of the ratio of the final count (N) to the starting (baseline) count (Xo), divided by the log of 2 (Greenwood et al., 2004a, b); that is

$$PD = [\log(N \div Xo)] \div \log 2.$$

Data is shown in Figure 3-5. Cells no longer in FGF-2 showed between 2-4 doublings from p5 to p9. However those cultures still supplemented with FGF-2 showed over 8 cell doublings over this time. This number of cell doublings is the equivalent of a difference of around 16 (2^4) times more cells produced over that culture period.

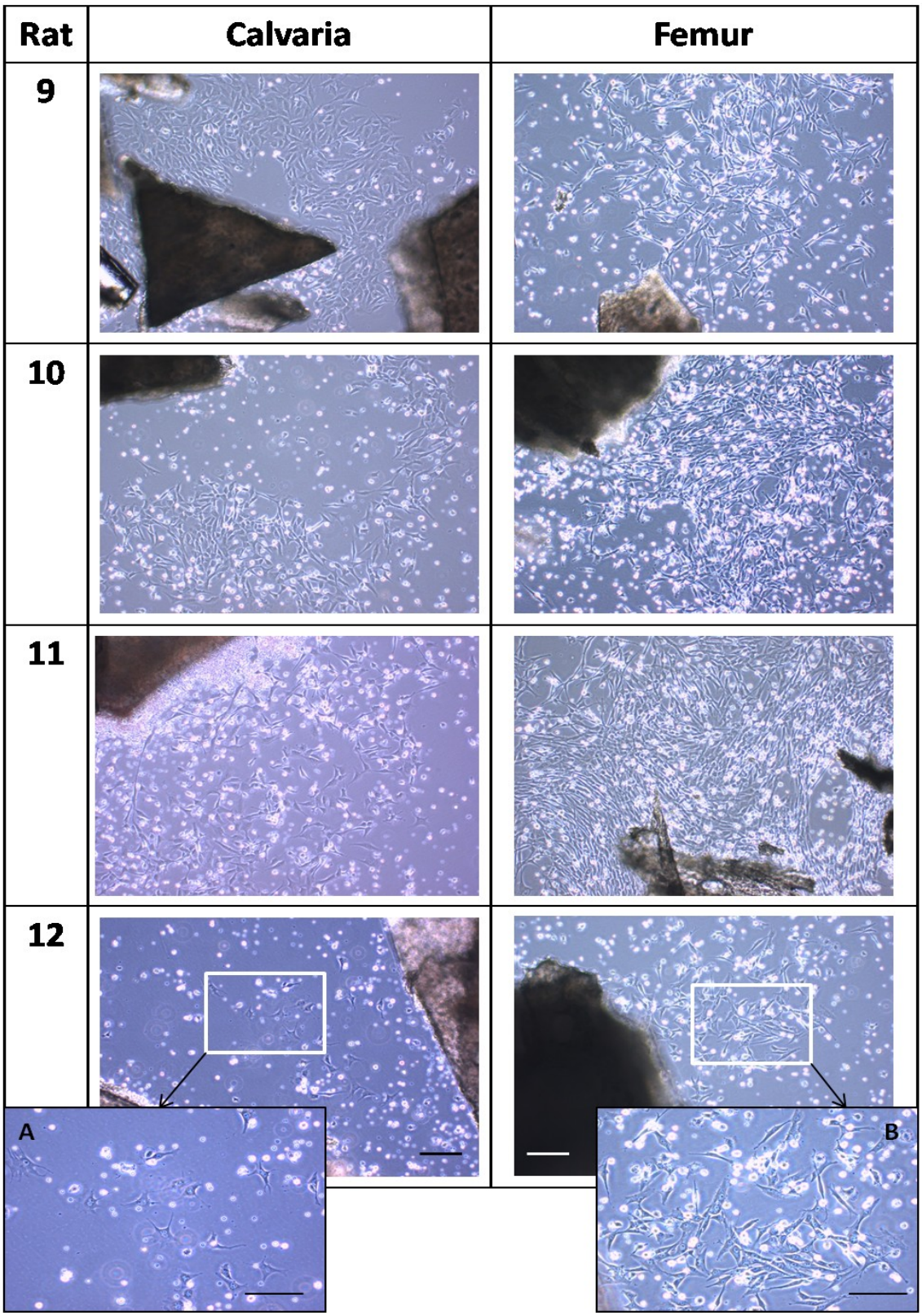


Figure 3-4: Four male rats (Rats 9-12) matched pairs at day 3 after explantation and grown in culture medium supplemented with FGF-2, marked outgrowths of osteoblastic cells were seen from both calvarial and femoral bone explants, magnification 40, scale bar = 100 µm. Cells derived from calvaria (A) tended to have a round or cuboidal shape, whilst femur-derived cells (B) tended to appear more spindle or long shape, at magnification x200, scale bar = 100 µm.

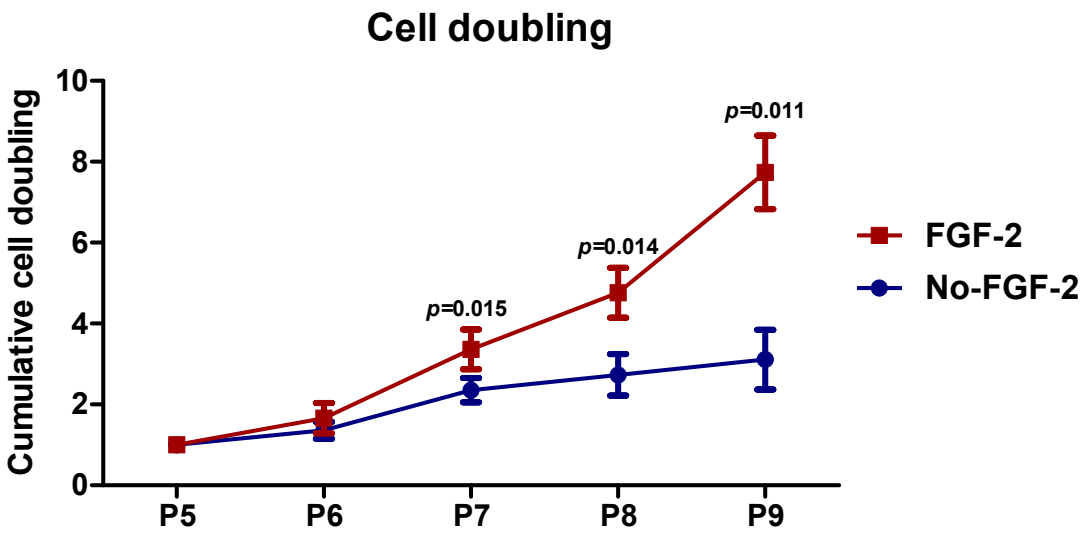


Figure 3-5: Cumulative cell doubling from adult rat calvarial and femoral osteoblastic cells from 4 lines, counted every 5 days using cell counter. The cells grown in osteoblast medium without FGF-2 supplementation showed between 2-4 cell doublings from p5 to p9, whilst cells grown in osteoblast medium supplemented with FGF-2 showed 8 cell doublings over this time. Data presented in mean ± SD. Paired *t*-test was used to compare cell doubling between osteoblasts grown in culture medium supplemented with FGF-2 and without FGF-2, $p<0.05$ considered significantly different.

3.4.3.1 ALP Staining

Rat calvarial osteoblastic cells (C-OBTs) and femoral osteoblastic cells (F-OBTs) from 4 male rats at passage 10 demonstrated stronger positive ALP staining when compared to BMSCs obtained and established from femoral bones of 4 rats. Rat dermal fibroblastic cells from scalp (S-FBTs), tissue overlying abdominal areas (Ab-FBTs) and femoral areas (Fe-FBTs) were used as negative controls and showed negative ALP staining. Rat Osteosarcoma (ROS 17/2.8) cells were used as a positive control reaction and showed strongest ALP staining (Figure 3-6). Microscopic examination showed strong ALP staining throughout the cultures in both calvarial and femoral osteoblastic cells at p5 (Figure 3-7) and p10 (Figure 3-8).

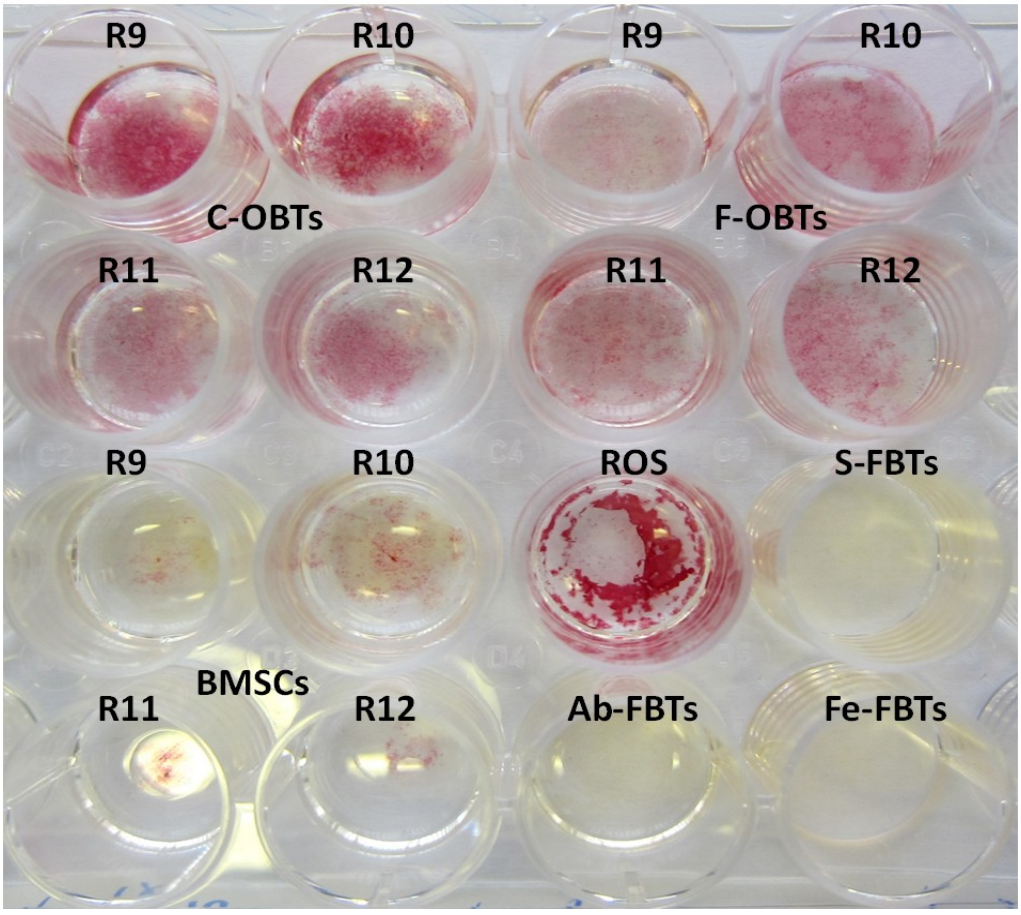


Figure 3-6: Rat calvarial and femoral osteoblastic cells from 4 rats (Rats 9-12) at passage 10 demonstrated stronger positive ALP staining as comparing to BMSCs, whilst rat dermal fibroblastic cells from scalp, tissue overlying abdominal and femoral areas were used as a negative control show negative ALP staining. ROS cells were used as a positive control show strongest ALP staining (C-OBTs = Calvarial osteoblastic cells, F-OBTs = Femoral osteoblastic cells, BMSCs = Bone Marrow Stromal Cells, ROS = Rat Osteosarcoma ROS 17/2.8 cells, S-FBTs = Dermal fibroblastic cells from scalp, Ab-FBTs = Dermal fibroblastic cells from tissue overlying abdominal area, Fe-FBTs = Dermal fibroblastic cells from tissue overlying femoral area).

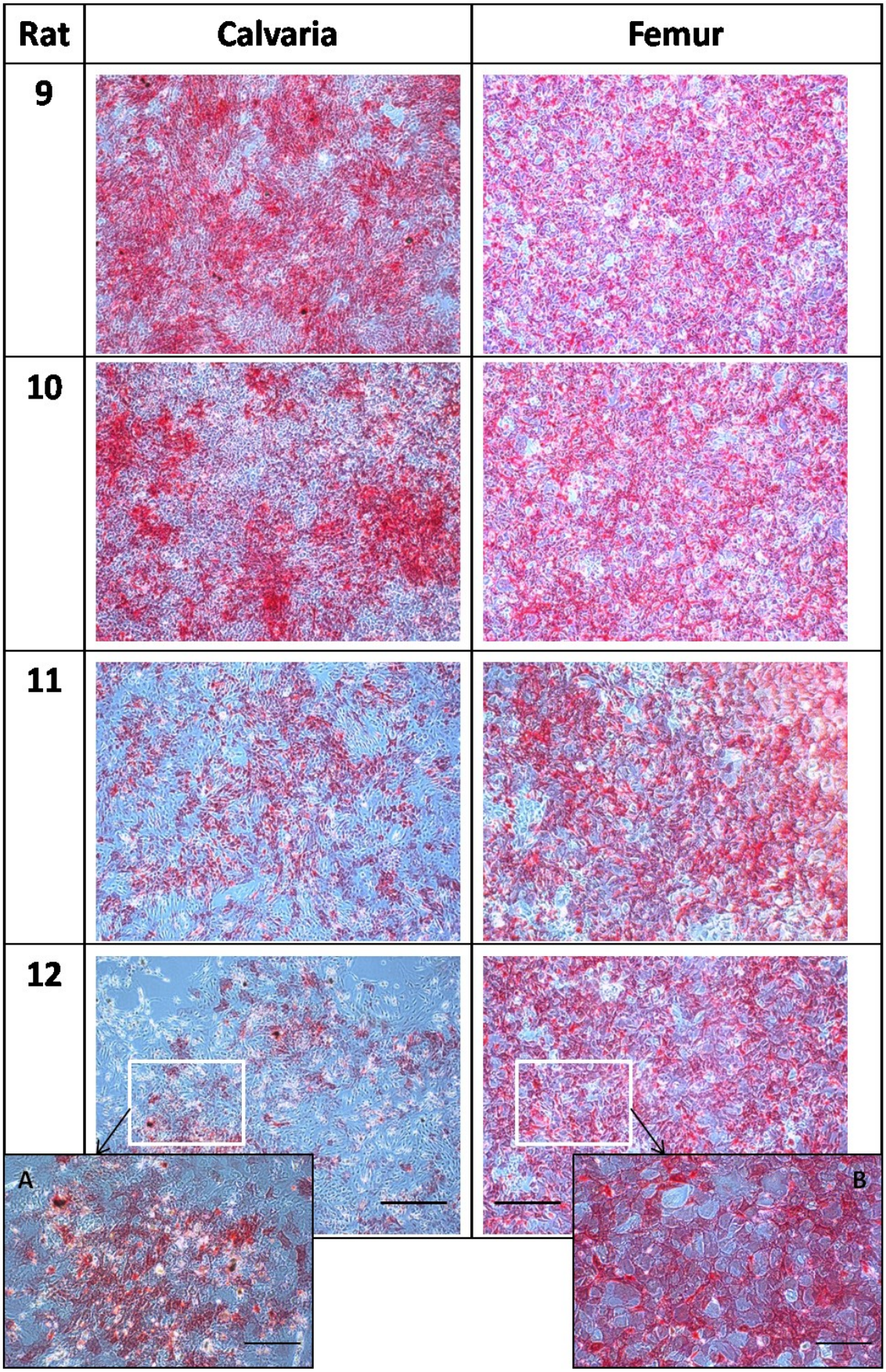


Figure 3-7: Rat calvarial and femoral osteoblastic cells from 4 male rats (Rats 9-12) at passage 5 showed positive ALP staining, original magnification x100, scale bar = 200 µm. A= R12C and B= R12F at magnification x200, scale bar = 100 µm. (R12C= Rat 12 calvaria, R12F= Rat 12 femur).

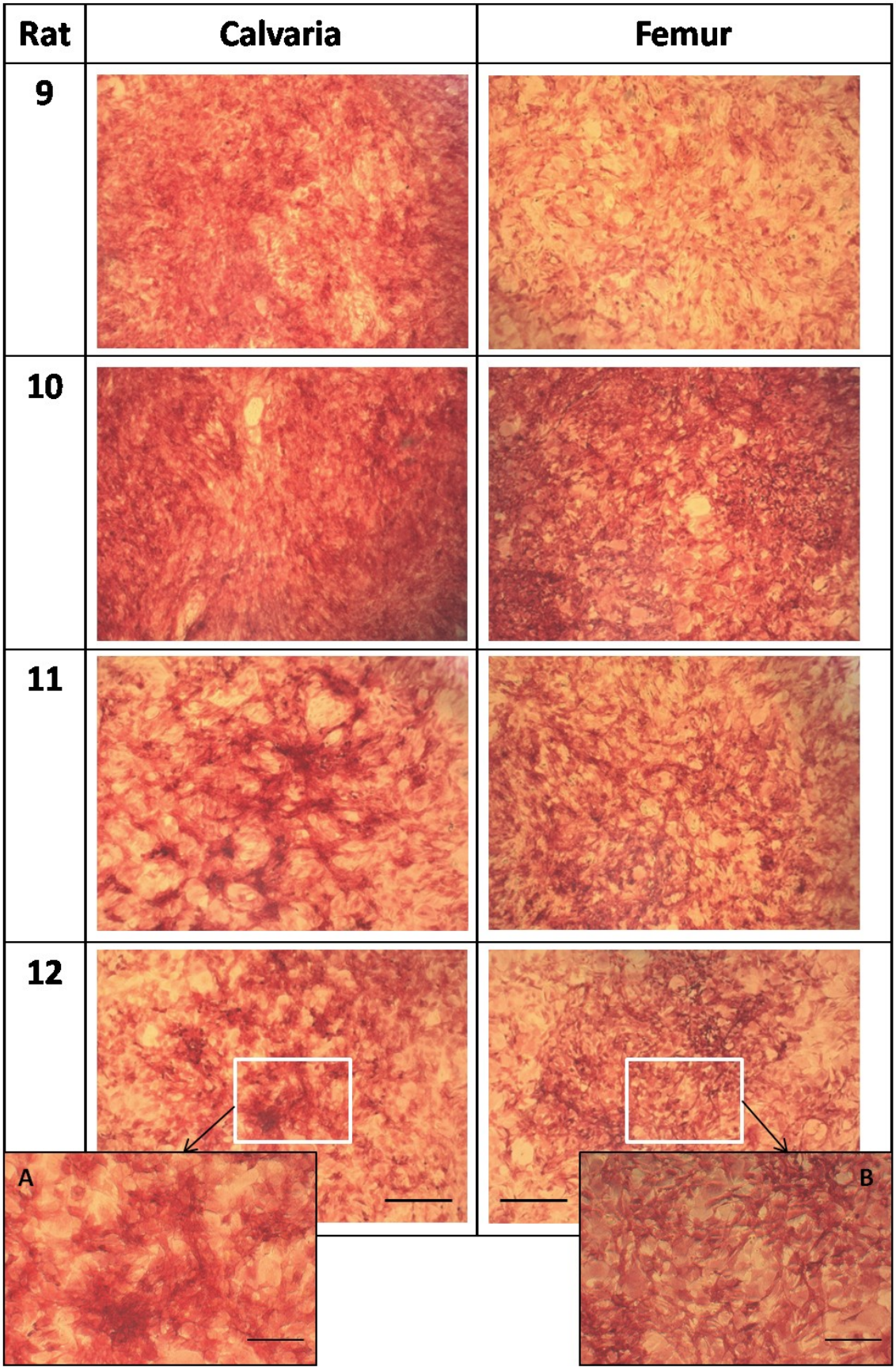


Figure 3-8: Rat calvarial and femoral osteoblastic cells from 4 male rats (Rats 9-12) at passage 10 showed positive ALP staining with stronger red colour due to more numbers of cells, magnification x100, scale bar = 200 µm. A= R12C and B= R12F at magnification x200, scale bar = 100 µm. (R12C= Rat 12 calvaria, R12F= Rat 12 femur).

3.4.3.2 Mineralised bone nodules

Cells at passage 10 were grown for 28 days in osteoblast differentiation medium, no FGF-2 supplemented, whilst supplemented with dexamethasone and β-glycerophosphate and stained with Alizarin red for bone nodules. Evidence of bone nodule formation was seen in all cultures although this appeared to have a reduced capacity to form nodules in calvarial osteoblast cultures (Figure 3-9).

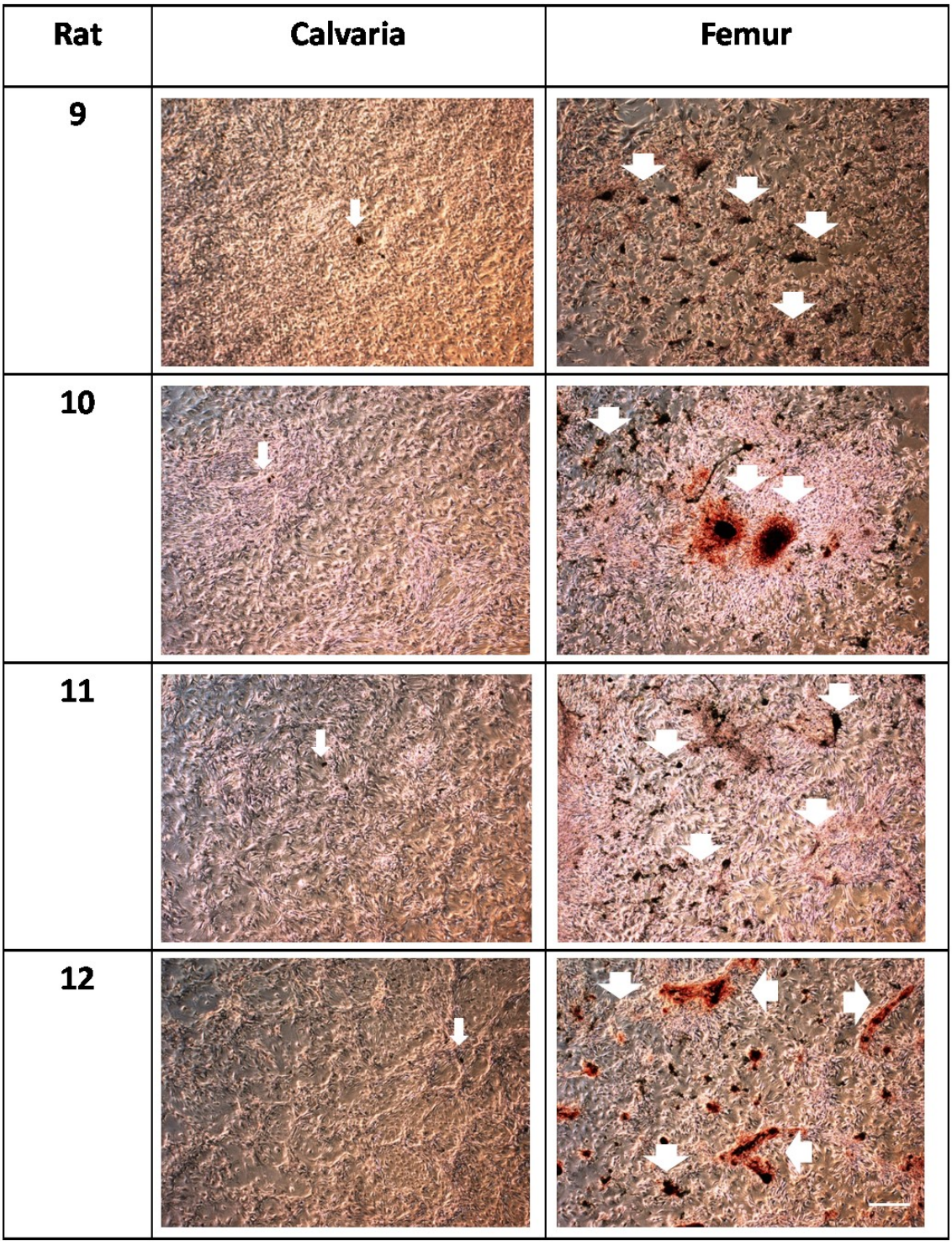


Figure 3-9: The primary osteoblast cultures were grown in osteoblast culture medium supplemented with FGF-2 up to p10. Then they were grown in osteoblast differentiation medium supplemented with dexamethasone and β-glycerophosphate, but no FGF-2 supplemented. At Day 28, mineralised bone nodules (arrows) stained with Alizarin red. The primary calvarial osteoblast cultures have less ability to form bone nodules (small arrows). Data represented from 4 male rats (Rats 9-12) at passage 10, magnification 40, scale bar = 100 μm.

3.4.3.3 Osteopontin production

Osteopontin production was seen by both calvarial and femoral osteoblastic cells at passage 10 (Figure 3-10). In contrast, rat dermal fibroblasts, used as negative controls, showed much lower osteopontin production. In general, femoral osteoblast cells showed higher osteopontin production than calvarial osteoblasts at both Day10 and Day14 and further investigation of osteopontin expression is described in Chapter 6.

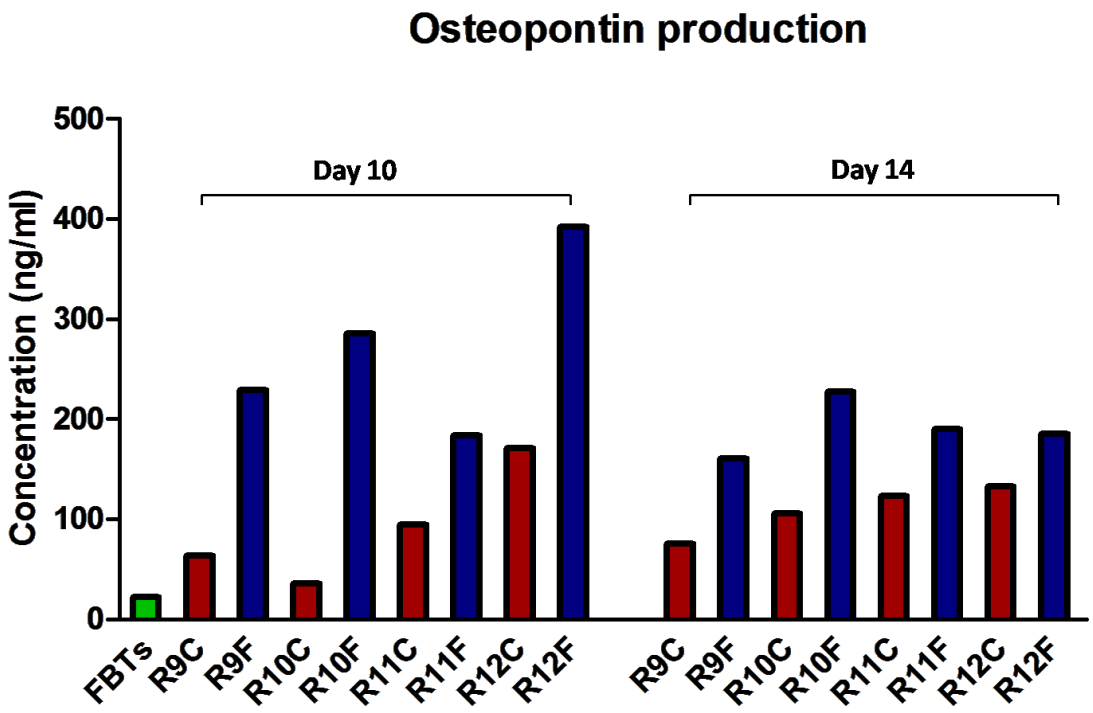


Figure 3-10: Osteopontin production (ng/ml) measured by ELISA. The calvarial and femoral derived cells were grown at passage 10 in medium supplemented with FGF-2 for 10 and 14 days. The cells were processed and collected for cell lysate. Data from 4 different male rats (Rats 9-12). Osteopontin production in rat dermal fibroblastic cells from scalp was derived from 1 rat as a negative control. 2 replicates were tested for each culture. Experiments were repeated 2 times and tested with 8 different primary osteoblast cultures each time. (FBTs = Dermal fibroblastic cells from scalp, R9C= Rat 9 calvaria, R10C= Rat 10 calvaria, R11C= Rat 11 calvaria, R12C= Rat 12 calvaria, R9F= Rat 9 femur, R10F= Rat 10 femur, R11F= Rat 11 femur and R12F= Rat12 femur).

3.4.3.4 Osteocalcin production

Low concentrations of osteocalcin measured by ELISA were also detected in the culture media from cells at passage 10 grown in osteoblast culture medium supplemented with FGF-2 for 14 days. There was no significant difference in osteocalcin production between calvarial osteoblastic cells and femoral osteoblastic cells ($p<0.05$) (Figure 3-11).

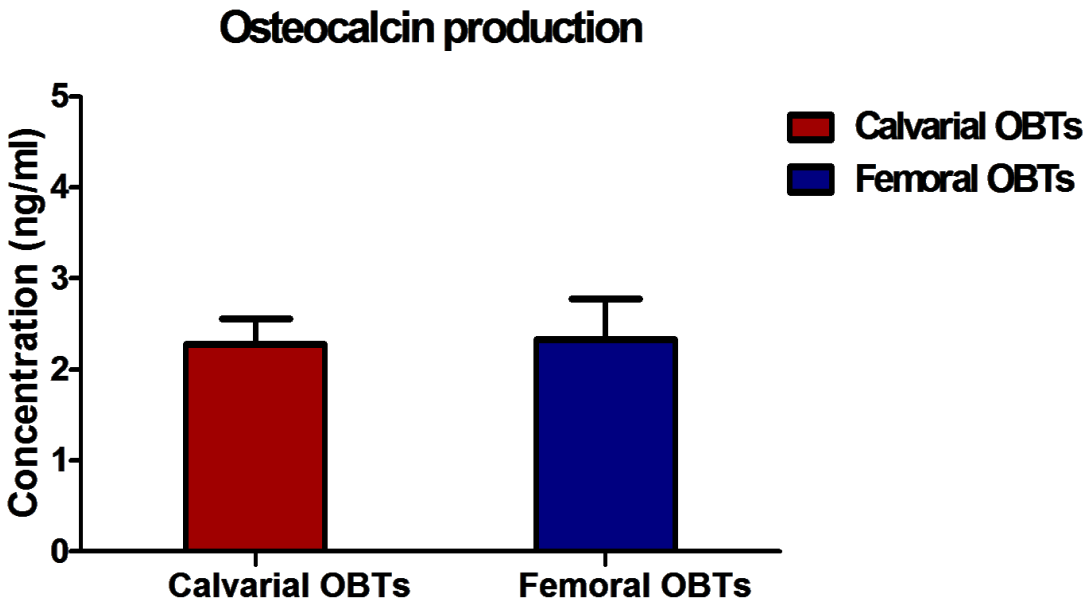


Figure 3-11: Osteocalcin production (ng/ml) measured by ELISA can be detected in rat calvarial and femoral derived cells at passage 10 grown in osteoblast culture medium supplemented with FGF-2. Data represented as mean ± SD from 4 different male rats (Rats 9-12). Data were generated from using 8 different primary osteoblast cultures and 2 replicates were tested for each culture. Paired *t*-test was used to compare osteocalcin production between two regionally distinct populations. This graph shows that osteocalcin can be detected in both cell types with no significantly difference ($p<0.05$). Three different experiments were repeated (data not shown).

3.4.3.5 Expression of osteoblast-associated genes

Osteoblastic cells at p10 were tested for expression of *Runx2*, *Bglap*, and *Spp1*. All lines showed expression of these osteoblast-associated genes, with some noticeable differences seen between femoral and calvarial osteoblasts. These data are described and discussed in details in Chapter 6.

3.5 Discussion and conclusion

A key requirement for the planned future experiments in this thesis was the establishment of primary osteoblast cultures derived from defined sites and from individual animals. Initial attempts to establish adult mouse and rat calvarial and femoral osteoblast cells were not successful using collagenase isolation and cell explant isolation methods. This was likely due to the small amounts of primary tissue available, unlike many studies using foetal mice or rats to establish primary cells successfully (Bellows et al., 1986; Deboni et al., 1996; Declercq et al., 2004).

A previous study compared extracts of human foetal and adult bones (40-year-old male) obtained by sequential demineralisation, on their effects on migration of osteoblast-like cells, demonstrated that extracts of human adult bones did not stimulate osteoblast migration and suggested that significant differences exist in protein composition and/or distribution between foetal and adult human bones (Somerman et al., 1983). The data suggested that human bone chemotactic activity decreased with advancing age and supported the clinical evidences where healing of bone fracture and bone remodeling occured at a much faster rate in children than adults and where the incidence of nonunion is comparatively rare in children; these differences in different age group may influence the potential of these bones remodelling (Somerman et al., 1983). Osteopontin expression was significantly reduced in more mature bone of young adult rats (Chen et al., 1993). Therefore, the age of the donor may be one factor that has influence on primary cell establishment *in vitro*.

We also observed that adult rat calvarial and femoral osteoblast cells had different morphologies when examined under the microscope as shown in Figure 3-4. The calvarial cells had a cuboidal shape, whilst femoral cells had a more spindle shape similar to other studies (Declercq et al., 2004; Nefussi et al., 1997).

A recent study demonstrated that calvarial osteoblastic cells of neural crest origin have better potential for osteogenic differentiation and displayed a higher capacity to undergo osseous healing compared with osteoblasts of paraxial mesoderm origin (Quarto et al., 2010). In frontal bone compared with parietal bone, endogenous canonical Wnt signalling was enhanced both *in vitro* and *in vivo* in neural crest-derived frontal bone

(Quarto et al., 2010). In paraxial mesodermal-derived parietal osteoblasts, the constitutive activation of canonical Wnt signalling could mimic the osteogenic potential of frontal osteoblasts, when canonical Wnt signalling in neural crest-derived frontal osteoblasts was knocked down, impaired osteogenic potential was observed (Quarto et al., 2010). The embryonic origin of the tissue makes a difference and active canonical Wnt signaling plays a major role in contributing to the superior intrinsic osteogenic potential and tissue regeneration in neural crest-derived frontal bone (Quarto et al., 2010).

In view of the persisting problems of establishing cultures we tested the effects of addition 10 ng/ml of Fibroblast growth factor-2 (FGF-2) to the osteoblast culture media. FGF-2 is an important modulator of cartilage and bone growth and differentiation (Montero et al., 2000). Bone formation is controlled by FGF signalling which regulating the expression of various genes involved in osteoprogenitor cell replication, osteoblast differentiation and apoptosis (Marie, 2003). FGF-2 is expressed and regulated in osteoblastic cells. The disruption of the *Fgf2* gene in mice results in decreased osteoblast replication, mineralised bone nodule formation, and new bone formation *in vivo* (Montero et al., 2000).

Based on our finding in this experiment, FGF-2 was supplemented in osteoblast culture media to overcome the problems of absence of growth in cultures not supplemented with FGF-2. In addition, primary osteoblast cultures showed positive ALP staining compared to primary fibroblast cultures (Figure 3-6). Our data suggested that FGF-2 did not inhibit ALP staining. In contrast, the previous study by Kalajzic et al., 2003 has shown that FGF-2 and noggin inhibited ALP in primary murine osteoblasts differentiation (Kalajzic et al., 2003) by observing alkaline phosphatase (ALP) and type I collagen expression (Col1a1) at day 7 (preosteoblast stage), bone sialoprotein (BSP) at day 11 (early osteoblast) and osteocalcin (OC) at day 15–18 (mature osteoblast stage). They have shown that FGF-2 completely inhibited expression of ALP and the mRNA transcript for Col1a1, while noggin showed only a partial inhibition of these markers of preosteoblast differentiation. However, the markers of differentiated osteoblasts (BSP and OC) were completely inhibited in both the FGF-2 and noggin treated cultures, suggested that noggin acts at later stage in the osteoprogenitor differentiation pathway than FGF-2. In addition, the collagen promoters driving green fluorescent protein

(GFP) that activate at different levels of osteoblast differentiation were analysed. The pOBCol3.6GFP and pOBCOL2.3GFP transgene activity was totally inhibited by continuous addition of FGF-2, while noggin showed partial inhibition of pOBCol3.6GFP and complete inhibition of the pOBCol2.3GFP transgene (Kalajzic et al., 2003). This work demonstrated that FGF-2 and noggin could reversibly modulate osteoblast lineage differentiation at different maturational stages (Kalajzic et al., 2003).

However, Quarto et al., 2010 recently reported that phosphorylation of GSK-3beta was induced by FGF-2 and the nuclear levels of beta-catenin in osteoblasts was increased, suggested that FGF might mediate and enhance activation of Wnt signalling (Quarto et al., 2010).

FGF-2 stimulation of osteoblast differentiation is partially through modulation of the Wnt/β-catenin pathway (Fei et al., 2011). The study on modulation of Wnt/β-catenin signaling using *Fgf-2*$^{-/-}$ mice by Fei and co-workers have shown that loss of endogenous FGF-2 resulted in reduced Wnt10b mRNAexpression, reduced *Lrp5*/*Lrp6* mRNAexpression, reduced β-catenin mRNAand protein expression, and reduced inactive phosphorylated GSK3β protein expression that led to reduced Wnt/β-catenin signalling (Fei et al., 2011). The reduction of Wnt/β-catenin signaling and Dkk2 mRNA expression which was important for terminal osteoblast differentiation probably contributed to decrease osteoblast differentiation and bone formation in *Fgf2*$^{-/-}$ mice (Fei et al., 2011). However, exogenous FGF-2 promoted β-catenin nuclear accumulation and induced inhibited glycogen synthase kinase-3β (GSK3β) which could increase Wnt signaling in *Fgf2*$^{-/-}$ BMSCs and probably contributed to rescue the decreased osteoblast differentiation in *Fgf2*$^{-/-}$ BMSCs (Fei et al., 2011).

In addition, FGF-2 induced calvarial osteoblast proliferation and enhanced cranial suture fusion, promoted rat osteoblast attachment and normal cell morphology as well as induced cell proliferation (Moursi et al., 2002). Wnt/β-catenin pathway directly regulated osteoblast transcription factors, including Runx2 and probably ATF4 (Fei et al., 2011). In addition, Runx2 is phosphorylated and activated by FGF-2 *via* the MAPK pathway suggested that FGF-2 plays an important role in regulation of Runx2 function and bone formation (Xiao et al., 2002).

In our study, supplementation of FGF-2 to the culture medium had a visibly marked effect on the cells resulting firstly in increased rate of proliferation, and secondly in maintenance of cultures through many passages, up to at least 10 passages here. One of the most important points to address about this culture system is whether despite the extended proliferation the cells still retained their osteoblastic phenotype. We therefore used a range of methods to test for osteoblast characteristics such as analysis of morphology, the presence of ALP activity, specific bone proteins (osteopontin and osteocalcin), production of mineralised nodules and expression of bone-associated genes such as *Runx2*, *Spp1* and *Bglap* (Deboni et al., 1996).

It was also apparent that there appeared to be significant differences in some of these phenotypic features between those cells derived from femurs and those from calvariae. We were able to show some osteoblast-associated genes such as *Runx2*, *Spp1* and *Bglap* in our primary osteoblast cultures by qRT-PCR and also demonstrate specific bone proteins such as osteopontin and osteocalcin using ELISA. These differences between calvarial and femoral osteoblasts are described and investigated further in Chapter 6.

In conclusion, the addition of FGF-2 to culture medium markedly stimulated proliferation and expansion of cells whilst retaining their osteoblastic phenotype for at least 10 passages as shown by positive ALP staining, mineralised bone nodule formation, production of osteopontin, but a low level of osteocalcin suggesting the FGF-2 helped proliferation, but not differentiation. FGF-2 supplementation was useful to enrich for and maintain a population of osteoprogenitor cells. The difference between two regionally distinct osteoblast cultures in mineralised bone nodule formation and osteopontin production may suggest the maintenance of positional identity that persists during embryonic body patterning and is still maintain in adult organism. This positional memory may effect on the plasticity in adult organisms and might also have an effect on bone regenerative capacity and regeneration.

Chapter 4

Chapter 4: Persistence of positional identity

4.1 Introduction

FGF signalling is initiated by ligand-dependent dimerisation of the FGFR which results in the cross phosphorylation of tyrosine residues of the receptor tyrosine kinase in the intracellular domain, then phosphorylated residues are bound specifically by several intracellular signal transduction proteins such as PLCγ, FRS2 and Src family members which start several intracellular signalling pathways, including the PLCγ pathway, PI3K/PKB pathway and the Ras/ERK pathway (Dorey and Amaya, 2010).

The cell responses to these different pathways are CamKII, calcium/calmodulin-dependent protein kinase II; DAG, diacylglycerol; ERK, extracellular-signal related kinase; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; FRS2, fibroblast growth factor receptor substrate 2; Gab, Grb2-associated protein; Grb2, growth factor receptor-bound protein 2; HSPG, heparan sulphate proteoglycan; IP3, inositol (1,4,5)-trisphosphate; MEK, mitogenactivated protein kinase kinase (also known as MAP2K); PI3K, phosphoinositide 3-kinase; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PKB, protein kinase B; PKC, protein kinase C; PLCγ, phospholipase C γ; cRaf, v-raf-leukemia viral oncogene homologue 1 (also known as RAF1); Ras, rat sarcoma (also known as Harvey rat sarcoma virus oncogene homologue); SHP2, SH2 domain-containing tyrosine phosphatase 2 (also known as PTPN11); SOS, son of sevenless; Src, sarcoma proto-oncogene tyrosine kinase (Dorey and Amaya, 2010).

During posterior body axis extension in mouse and chick embryos, FGF signalling is high and can maintain an undifferentiated state at the posterior end. The ectoderm and mesoderm differentiation promoted by Retinoic acid (RA), the FGF antagonised by an inverse RA signalling, the RA signalling expresses over FGF signalling, the overlapping process of differentiation of neural plate, undifferentiated presomitic mesoderm (PSM), differentiated somitic mesoderm, and somatic mesoderm was evidenced.

Wnt8c is expressed in the neural plate, whereas FGF-8 and Raldh2 are expressed in the mesoderm, and signal across germ layers (Dorey and Amaya, 2010). FGF signalling preferentially regulates the 5' (more-posterior) *Hox* genes, whereas RA signalling preferentially regulates the 3' (more-anterior) *Hox* genes (Bel-Vialar et al., 2002). In

addition, FGF-2 and FGF-8 seem to play an important role in promoting proliferation and differentiation of cranial neural crest, but not trunk neural crest cells and that depends at least in part on the expression of trunk Hox genes (Abzhanov et al., 2003). For normal patterning of the 2nd branchial arch, *Hoxa2* expression was required for cranial neural crest cells fate to become cartilage (Abzhanov et al., 2003).

The study of *Hox* genes expressed in the adult was carried out in individual CFU-F colonies derived from various organs and anatomical locations using hierarchical cluster analysis has revealed *Hox* expression are heterogeneous but highly specific for their anatomical origin and the *Hox* codes is maintained during differentiation suggested that they originate from anterioposterior axis, but may not originate from prevertebral mesenchyme and showed consistentcy with a role for Hox proteins in specifying cellular identity of MSC (Ackema and Charite, 2008).

As previously discussed, Rawlinson and co-workers have recently demonstrated that regional gene expression profiles from different bones had significant differences. Furthermore they went on to show that isolated osteoblasts from these bones continued to show distinct gene expression profiles (Rawlinson et al., 2009b). In particular one of the striking differences between cells derived from different anatomical sites was the expression of a range of markers associated with their embryonic origin. Thus in that study skull bone-derived cells preferentially expressed genes reflecting their neural crest origin such as *Dlx* and *Msx2*, whilst limb bone-derived cells expressed *Hox, Shox2* and *Tbx* genes. They further investigated *Hoxa* gene expression profiles in adult bone-derived cells in long bones and from the rib. Differential *Hoxa* expression profiles (*Hoxa2*, *Hoxa5*, *Hoxa7* and *Hoxa10*) were clearly evident in endochondral/mesoderm bones, whilst *Hoxa13* was present only in the ulna and femur samples.

In keeping with the results of these studies, Rinn and co-workers reported that the *Hox* gene profile in cultured human skin fibroblasts demonstrated specific and distinct expression patterns depending on the sites where those mesoderm-derived cells were established from (Rinn et al., 2006; Rinn et al., 2008a).

The persistence of homeobox gene expression in the adult, as described in the studies above, may reflect positional information during embryogenesis and suggests that cells

maintain a memory of their embryonic origin or body positioning. Alternatively, these observations may indicate that the local environment of the cell is able to specify its positional identity. In support of this option, the study of Leucht and co-workers used a transplantation assay to address the question of whether the embryonic origin of the graft influenced its cell fate (Leucht et al., 2008a). They found that mesoderm-derived cells did not appear to demonstrate the same plasticity as neural crest-derived cells when they were transplanted into mandibular defects. The grafted mesodermal cells becoming differentiated chondrocytes even though the environment fully supported osteogenic differentiation *in vivo* (Leucht et al., 2008a). They suggested that there was a fundamental difference in the plasticity of neural crest-derived and mesoderm-derived progenitor cells.

All of these observations may have significant physiological and possibly clinical implications. The regulation of osteoblast behaviour at different sites may be different and systemic hormonal changes may have different effects on different skeletal sites. For example the maintenance of bone density in long bones is dependent on mechanical loading and is susceptible to post-menopausal osteoporosis, whereas maintenance of cranial bone is neither mechanical load dependent nor susceptible to osteoporosis.

Previous studies have different opinions. One group supported the idea that the local environment played a role in cell modulation (Leucht et al., 2008a). In contrast, the other group supported the idea that the cells have already been committed, pre-programmed and retained their positional identity even they have been detached from their *in vivo* environment to an *in vitro* environment (Rawlinson et al., 2009b).

In order to explore the likely explanation for expression of positional identity markers, the aim of the studies described in this chapter is to investigate the stability and persistence of *Hoxa* gene expression patterns of regionally distinct osteoblasts when maintained in culture, removed from their normal environment *in vivo*. Specifically, the experiments will test the hypothesis that the patterns of *Hoxa* gene expression from regionally distinct osteoblastic cells are stable in long term culture.

In addition to looking at *Hoxa* gene expression pattern, we have also looked at some transcription factors that are differentially expressed embryonic markers. Rawlinson

and co-workers have found that *Msx2* and *Irx5* preferentially expressed in skull bone-derived cells, whilst *Tbx3* preferentially expressed in limb bone-derived cells (Rawlinson et al., 2009b).

That is why we investigated further the results of Rawlinson and co-workers' study in depth to investigate whether the *Hoxa* gene can be maintained in cultures for long period of time and can be modulated to enhance osteogenic potential and improve bone regenerative capacity for bone grafting and tissue engineering or not.

4.2 Aims

The aims of this chapter are:

1. To investigate the stability and persistence of *Hoxa* gene expression patterns of regionally distinct osteoblasts when maintained in culture;

2. To investigate whether the patterns of *Hoxa* gene expression from regionally distinct osteoblastic cells are stable in long term culture or not;

3. To investigate the transcription factors those are expressed in osteoblastic cells from distinct embryonic origin.

4.3 Materials and Methods

4.3.1 Cell cultures

Initial pilot experiments were carried out on rat dermal fibroblasts derived from different anatomical sites and cultured in fibroblast culture medium as described in section 2.2.3. When the cells were 80-90% confluent, the cultures were passaged and continued to grow up to passage 5, at which time cells were tested for their *Hoxa* gene expression profile.

Rat bone marrow stromal cells (BMSCs) derived from different bony sites (humerus, tibia, femur, ulna and rib) were kindly provided by Dr Ian McKay. Cells were originally established by aspirating bone marrow from medullary cavities of bones as previously described (Maniatopoulos et al., 1988). Cells were grown until passage 3 and then the samples were tested for their *Hoxa* gene expression profile. Cells were derived from 5 different animals.

The calvarial and femoral osteoblast cell cultures from 4 male rats were obtained by explants and collagenase digestion methods as described in section 2.2.3. The cells were cultured in osteoblast culture medium supplemented with 10 ng/ml of FGF-2. When the cells reached 80-90% confluence, the cultures were passaged and continually cultured up to passage 5 and 10, when cultures were stopped, and samples taken for analysis of their *Hoxa* gene expression profile.

4.3.2 RNA extraction, cDNA synthesis and qRT-PCR

The cells were collected for RNA extraction, cDNA synthesis and assessed for *Hoxa* gene expression and transcription factors (*Msx2*, *Irx5* and *Tbx3*) using qRT-PCR. The details have been described in sections 2.12-2.18 and Table 4-1.

Sample	Method	Gene	Array	qRT-PCR
Cells	Illumina	*Msx2*	13.10	11.40
		Irx5	16.10	6.95
		Tbx3	0.13	0.41

Table 4-1: Validation of gene array with qRT-PCR from bone-derived cells, data shown in Skull/Limb ratio. *Msx2* and *Irx5* preferentially expressed by skull bone-derived cells, whilst *Tbx3* preferentially expressed by limb bone-derived cells. Adapted from: (Rawlinson et al., 2009b).

4.4 Results

4.4.1 Dermal fibroblasts

4.4.1.1 Initial experiments

Dermal fibroblasts were isolated from one female Wistar rat, from explants of 4 different sites: scalp, tissue overlying shoulder, abdominal and ulna area. The cells were expanded and passaged in culture. After initial plating the non-adherent cells were removed and cells were cultured until they reached 80-90% confluence. Cultures of rat dermal fibroblasts were then lysed and total RNA extracted. Gene expression was assessed by qRT-PCR (Taqman) to investigate levels of *Hoxa1*, *Hoxa2*, *Hoxa4*, *Hoxa5*, *Hoxa7*, *Hoxa10* and *Hoxa13*.

Distinct patterns of *Hoxa* gene expression between the different cultures (Figures 4-1-4). Firstly no expression of *Hoxa* genes was seen in cells derived from the scalp. *Hoxa1* expression was highest in rat dermal fibroblasts derived from tissue overlying shoulder area (Figure 4-1). *Hoxa4* showed greatest expression in rat dermal fibroblasts derived from tissue overlying abdominal area (Figure 4-2). No expression of *Hoxa5* was seen in cells from either the scalp or the shoulder. *Hoxa5* showed greatest expression in cells from tissue overlying abdominal area (Figure 4-3).

No expression of *Hoxa7* was seen in cells from scalp or the shoulder. *Hoxa7* showed greatest expression in cells from tissue overlying ulna area (Figure 4-4). A summary of the *Hoxa* gene expression profiles is shown in Table 4-2.

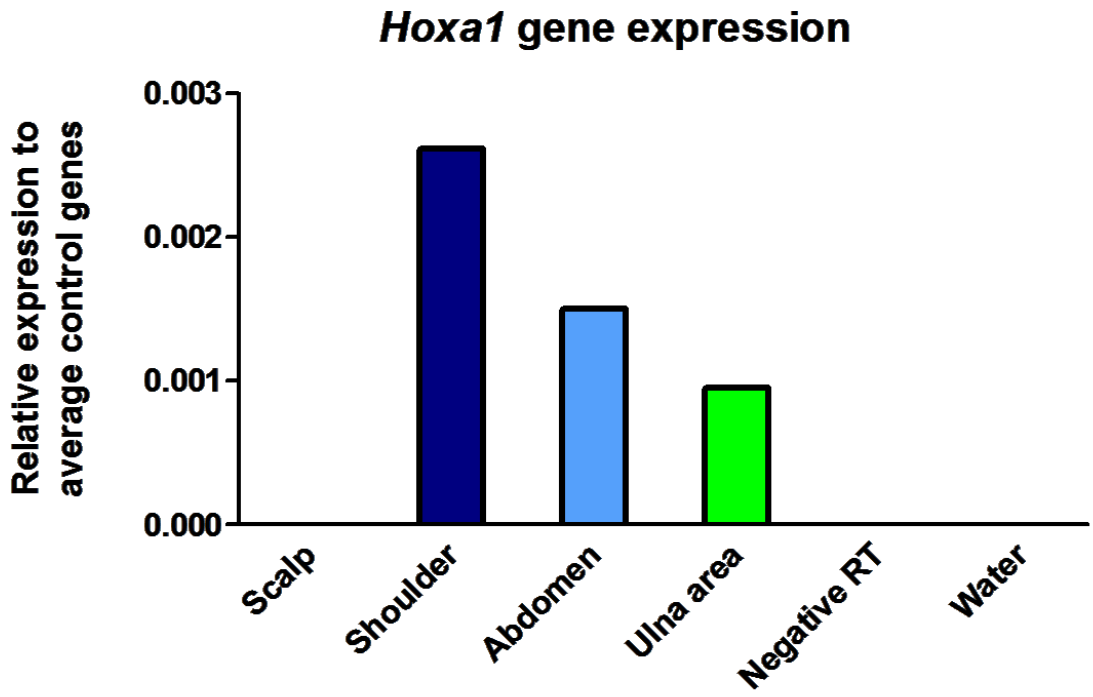


Figure 4-1: Relative expression of *Hoxa1* gene to average control genes (*Eif4a2* and *UBC*) in rat dermal fibroblasts derived from 4 different sites. Data represented from one female rat and 4 replicates were tested for each culture. This showed no expression of *Hoxa1* in rat dermal fibroblasts derived from scalp. *Hoxa1* expressed highest in shoulder region.

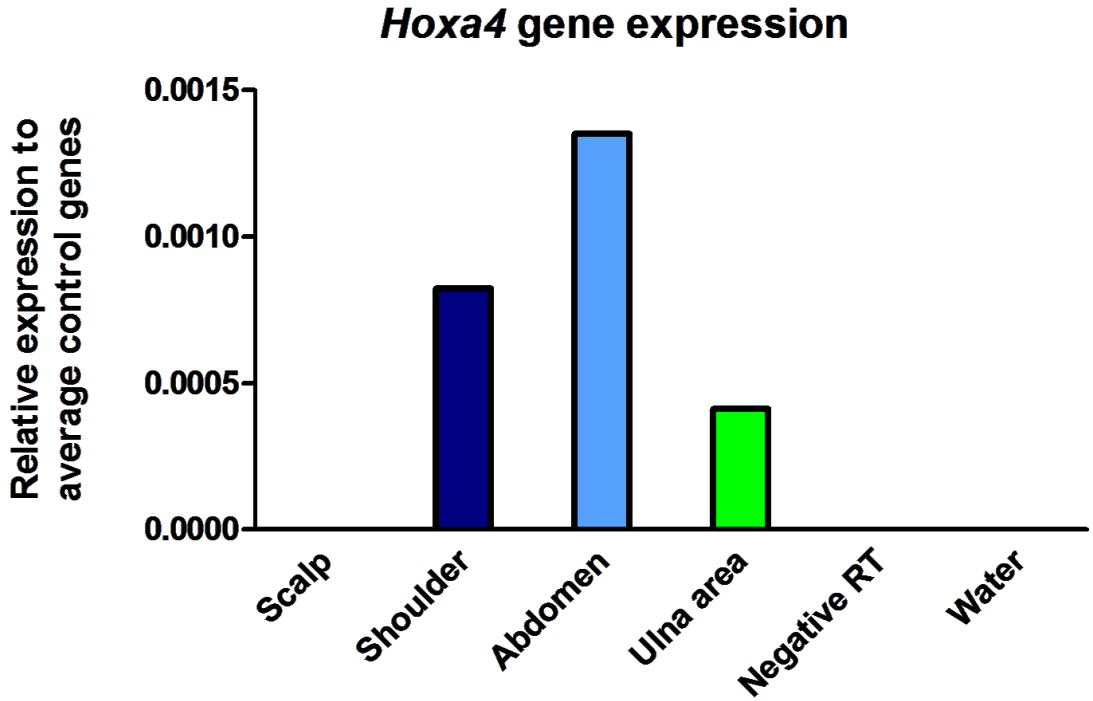


Figure 4-2: Relative expression of *Hoxa4* gene to average control genes (*Eif4a2* and *UBC*) in rat dermal fibroblasts derived from 4 different sites. Data represented from one female rat and 4 replicates were tested for each culture. This showed no expression of *Hoxa4* in rat dermal fibroblasts derived from scalp. *Hoxa4* expressed highest in abdominal region.

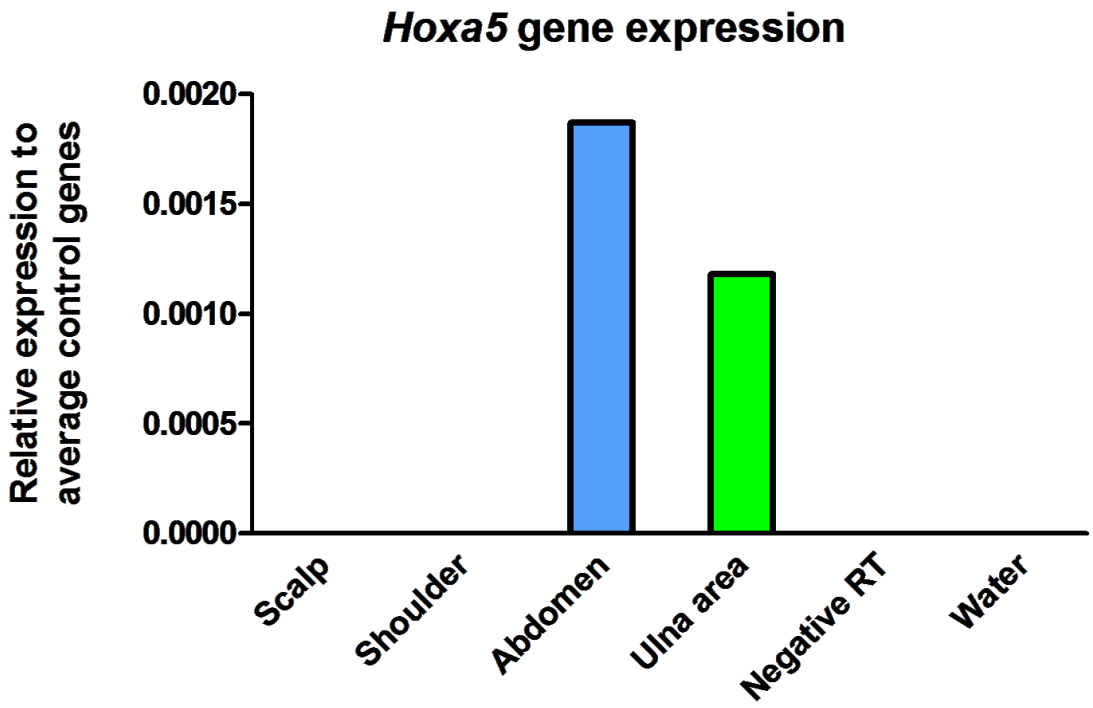


Figure 4-3: Relative expression of *Hoxa5* gene to average control genes (*Eif4a2* and *UBC*) in rat dermal fibroblasts derived from 4 different sites. Data represented from one female rat and 4 replicates were tested for each culture. This showed no expression of *Hoxa5* in rat dermal fibroblasts derived from scalp and shoulder regions. *Hoxa5* expressed highest in abdominal region.

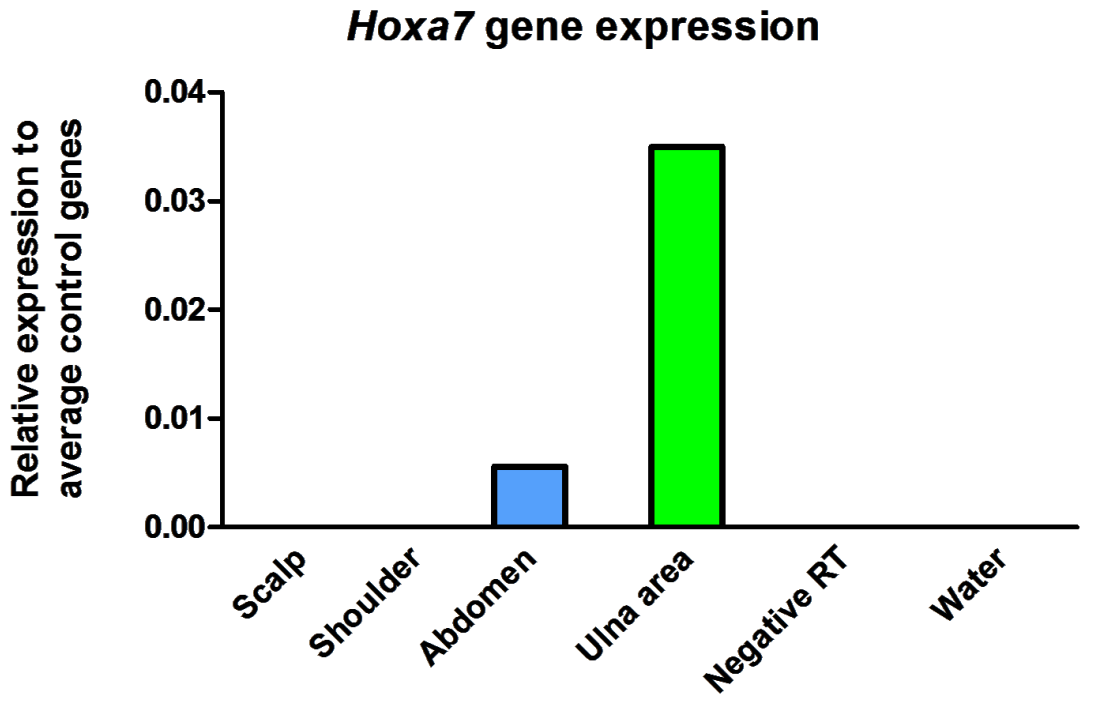


Figure 4-4: Relative expression of *Hoxa7* gene to average control genes (*Eif4a2* and *UBC*) in rat dermal fibroblasts derived from 4 different sites. Data represented from one female rat and 4 replicates were tested for each culture. This showed no expression of *Hoxa7* in rat dermal fibroblasts derived from scalp and shoulder regions. *Hoxa7* expressed highest in ulna region.

Region	*Hoxa* gene expression
Scalp	*Hoxa* negative
Shoulder	*Hoxa1*
Abdomen	*Hoxa2, Hoxa4, Hoxa5, Hoxa13*
Ulna area	*Hoxa7, Hoxa10*

Table 4-2: Summary of *Hoxa* gene expression in rat dermal fibroblasts from tissue overlying different regions in the same animal.

In this preliminary experiment *Hoxa* gene expression profile was tested in rat dermal fibroblasts and was useful to optimise our system before further study in primary osteoblast cultures. The data showed the expression roughly followed colinearlity. For example, the most anterior part of the body showed no *Hoxa* gene expression i.e. scalp, whilst the more posterior parts expressed a greater number of *Hoxa* genes. The pattern of *Hoxa* gene expression which clearly evidenced in embryo was also evidenced in adult organism. It was still maintained in fully developed animals.

4.4.1.2 Further experiments with dermal fibroblasts

On completion of the initial pilot study a further experiment was carried out with fibroblasts derived from 3 different anatomical sites (scalp, tissue overlying abdominal and femoral areas) from 4 rats, which had been established and grown in growth media up to passage 5. Then the cells were collected for RNA extraction, cDNA synthesis and assessed for *Hoxa* gene expression by qRT-PCR as before.

We have found that there is a pattern of *Hoxa* gene expression at passage 5 in culture. There is no *Hoxa* gene expression in rat dermal fibroblasts derived from scalp (see Appendix 4), whilst *Hoxa7* and *Hoxa10* are highly expressed in dermal fibroblasts derived from tissue overlying femoral area (Figure 4-5).

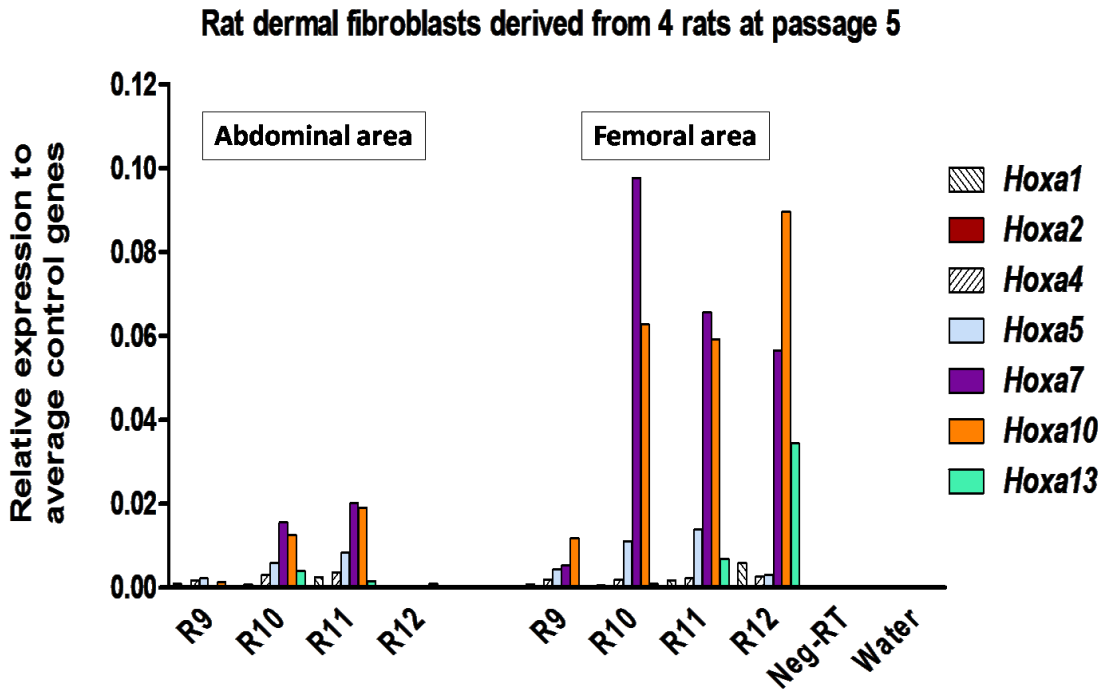


Figure 4-5: Relative expression of *Hoxa* genes to average control genes (*Eif4a2* and *UBC*) in rat dermal fibroblasts derived from 3 different sites; scalp, tissue overlying abdominal and femoral areas from 4 male rats (Rats 9-12). Data were generated from using 12 different primary osteoblast cultures and 4 replicates were tested for each culture. This showed a pattern of *Hoxa* gene expression in cultures at passage 5. No *Hoxa* gene expression seen in rat dermal fibroblasts derived from scalp (see Appendix 4), *Hoxa7* and *Hoxa10* highly expressed in rat dermal fibroblasts derived from tissue overlying femoral area (R9= Rat 9, R10= Rat 10, R11= Rat 11, R12= Rat 12, and Neg-RT= Negative RT).

4.4.2 Bone Marrow Stromal Cells

Expression of *Hoxa* genes was detectable in all cultures (Figure 4-6). The pattern of *Hoxa* gene expression in the rib was distinct from other bones, showing highest expression of *Hoxa4* and *Hoxa5* and low levels of *Hoxa7*. Patterns of expression of marrow from the femur, humerus and tibia were similar, with expression of *Hoxa7* most abundant and slightly lower levels of *Hoxa10*. Most notably, expression of *Hoxa13* was restricted to the ulna (Figure 4-6). Levels of *Hoxa1* were uniformly low, whilst levels of *Hoxa2* were extremely low - close to detection limits in all cultures (Figure 4-6).

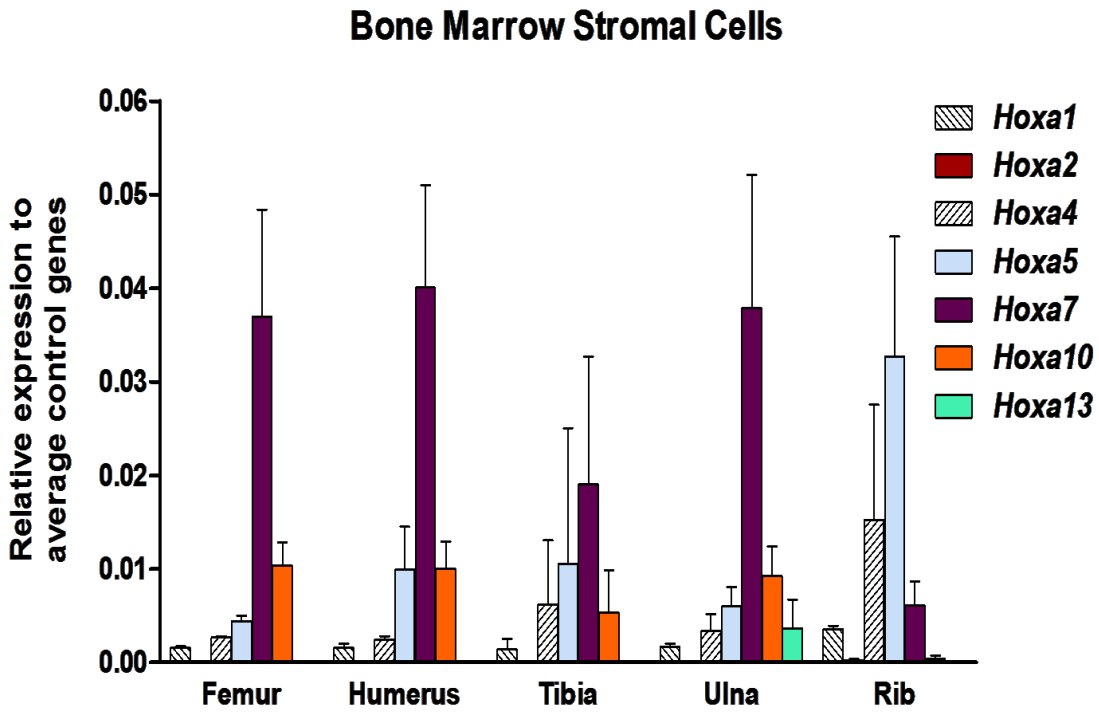


Figure 4-6: Relative expression of *Hoxa* genes to average control genes (*Atp5b* and *Eif4a2*) in BMSCs derived from 5 different sites; femur, humerus, tibia, ulna and rib. Data represented as mean ± SD from 5 different male rats, 4 replicates were tested for each culture. This showed a pattern of *Hoxa* gene expression in BMSCs cultures at passage 3. *Hoxa2* gene expression was extremely low, nearly undetectable seen in all cultures, *Hoxa7* and *Hoxa10* highly expressed in all cultures, except in rib which showed different pattern and expressed *Hoxa4* and *Hoxa5* highest. Interestingly, *Hoxa13* only expressed in ulna.

Expression of *Hoxa5* genes was detectable in all BMSCs cultures. The highest expression of *Hoxa5* expression was seen in the rib-derived cells, which was significantly different (**, $p<0.01$) from other sites (Figure 4-7).

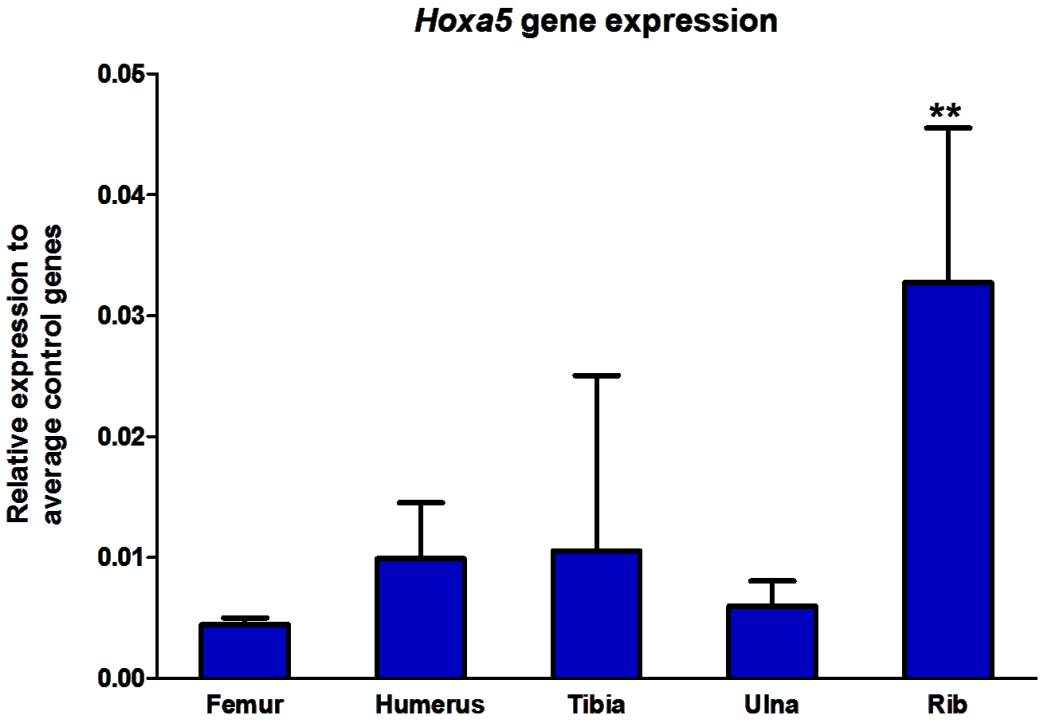


Figure 4-7: Relative expression of *Hoxa5* gene to average control genes (*Atp5b* and *Eif4a2*) in BMSCs derived from 5 different sites; femur, humerus, tibia, ulna and rib. Data represented as mean ± SD from 5 different male rats, 4 replicates were tested for each culture. *Hoxa5* expression in rib was significantly different to others sites at **, $p<0.01$ using One-way ANOVA and Bonferroni's Multiple Comparison Test.

In addition, the expression of *Hoxa7* genes was also detectable in all BMSCs cultures. *Hoxa*7 expression in tibia and rib were significantly different to others sites (**, $p<0.01$), but tibia and rib were not significantly different from each other (Figure 4-8).

Figure 4-8: Relative expression of *Hoxa7* gene to average control genes (*Atp5b* and *Eif4a2*) in BMSCs derived from 5 different sites; femur, humerus, tibia, ulna and rib. Data represented as mean ± SD from 5 different male rats, 4 replicates were tested for each culture. *Hoxa*7 expression in tibia and rib were significantly different to others sites, but tibia and rib were not significantly different to each other at **, $p<0.01$ using One-way ANOVA and Bonferroni's Multiple Comparison Test.

Interestingly, the expression of *Hoxa13* genes was detectable in all BMSCs cultures. *Hoxa*13 expression was highest in BMSCs derived from the ulna region. *Hoxa*13 expression in ulna and rib were significantly different to each other (***, $p<0.001$) (Figure 4-9).

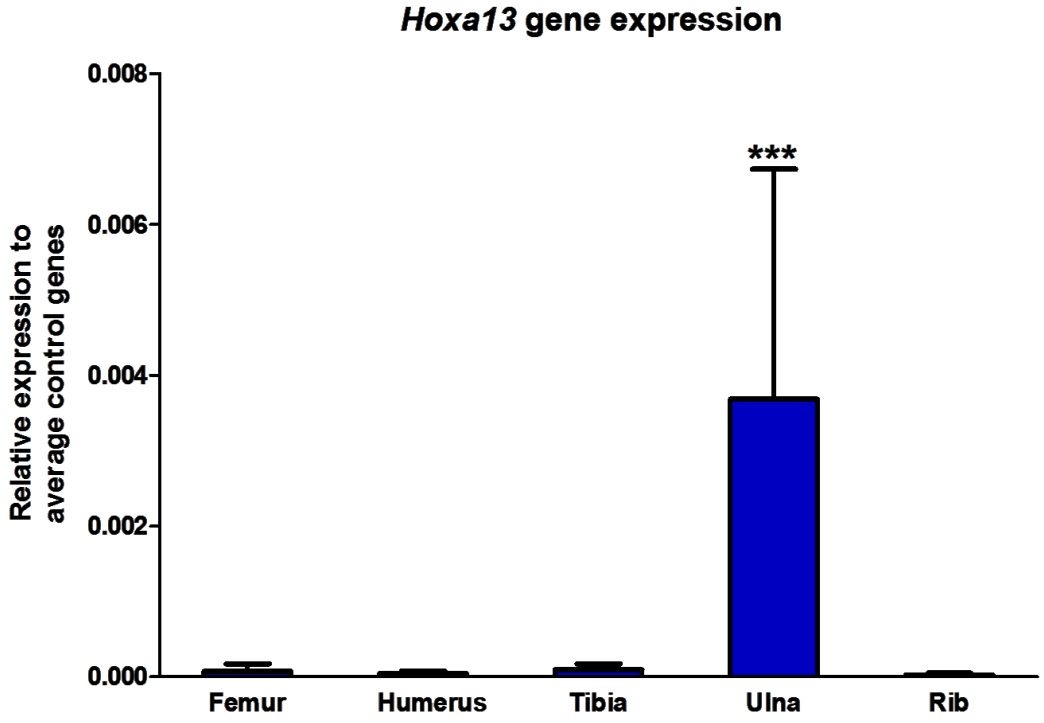


Figure 4-9: Relative expression of *Hoxa13* gene to average control genes (*Atp5b* and *Eif4a2*) in BMSCs derived from 5 different sites; femur, humerus, tibia, ulna and rib. Data represented as mean ± SD from 5 different male rats, 4 replicates were tested for each culture. *Hoxa*13 expression in ulna and rib were significantly different to each other at ***, $p<0.001$ using One-way ANOVA and Bonferroni's Multiple Comparison Test.

However, *Alp*, *Bglap* and *Tgfbi* gene expression were not significantly different to each others among 5 different sites (Figure 4-10 a-c).

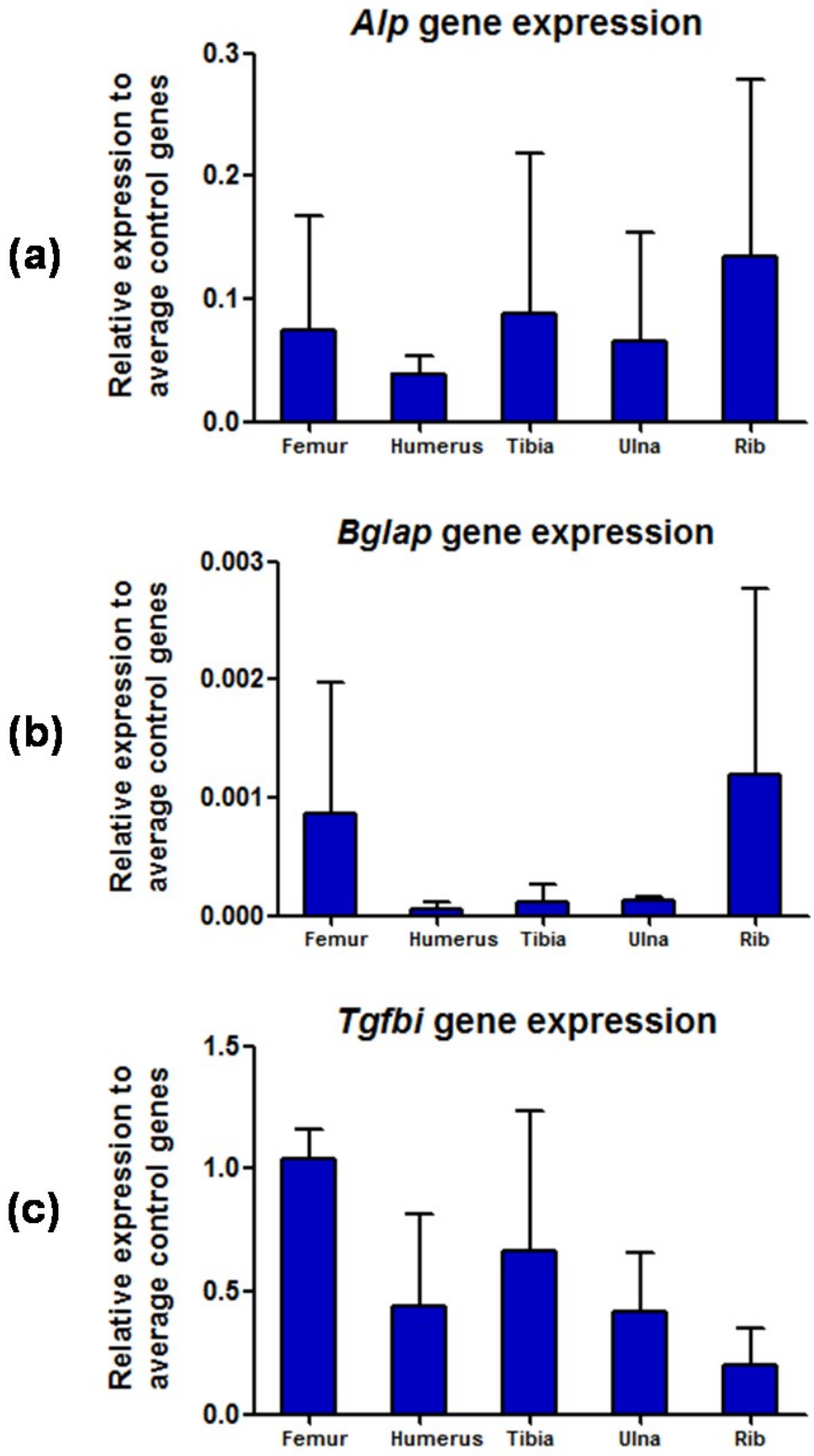


Figure 4-10: BMSCs from 5 different sites were tested for; (a) *Alp* gene expression; (b) *Bglap* gene expression; (c) *Tgfbi* gene expression. *Alp*, *Bglap* and *Tgfbi* expression among these 5 different sites were not significantly different to each others at $P<0.05$ using One-way ANOVA and Bonferroni's Multiple Comparison Test.

4.4.3 Osteoblastic cells

4.4.3.1 *Hoxa* gene expression

Rat calvarial and femoral osteoblastic cells from 4 male rats were tested for *Hoxa* gene expression at passage 5 and at passage 10. There was no *Hoxa* gene expression in calvaria-derived osteoblast cultures seen at either passage 5 or passage 10 (see Appendix 5). The femur-derived cells from the 4 different rats all showed a similar pattern of *Hoxa* gene expression at passage 5 and passage 10 (Figures 4-11 and 4-12). Most notably *Hoxa7* expression was highest in all femoral osteoblast cultures.

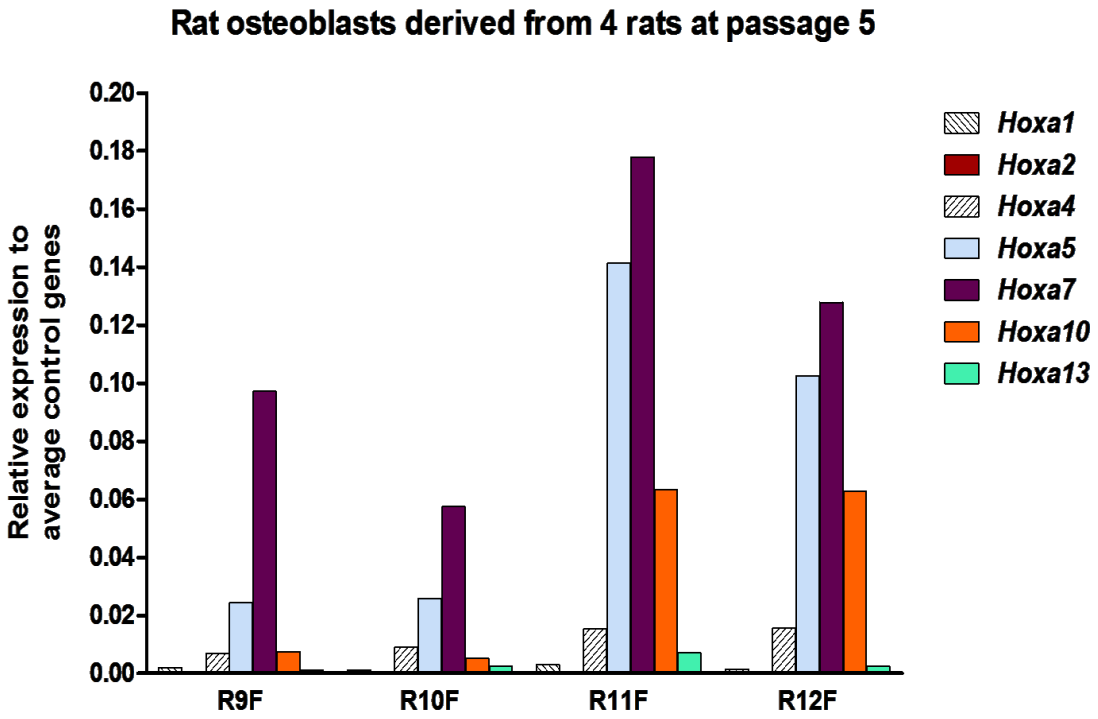


Figure 4-11: Relative expression of *Hoxa* genes to average control genes (*Atp5b* and *Eif4a2*) in adult rat calvarial and femoral osteoblastic cells from 4 male rats (Rats 9-12) matched pairs. Data were generated from using 8 different primary osteoblast cultures and 4 replicates were tested for each culture. There was no *Hoxa* gene expression in calvarial osteoblastic cells (see Appendix 5). This showed pattern of *Hoxa* gene expression at passage 5. *Hoxa5* and *Hoxa7* gene expressed highest in all cultures of femoral osteoblastic cells (R9F= Rat 9 femur, R10F= Rat 10 femur, R11F= Rat 11 femur and R12F= Rat12 femur).

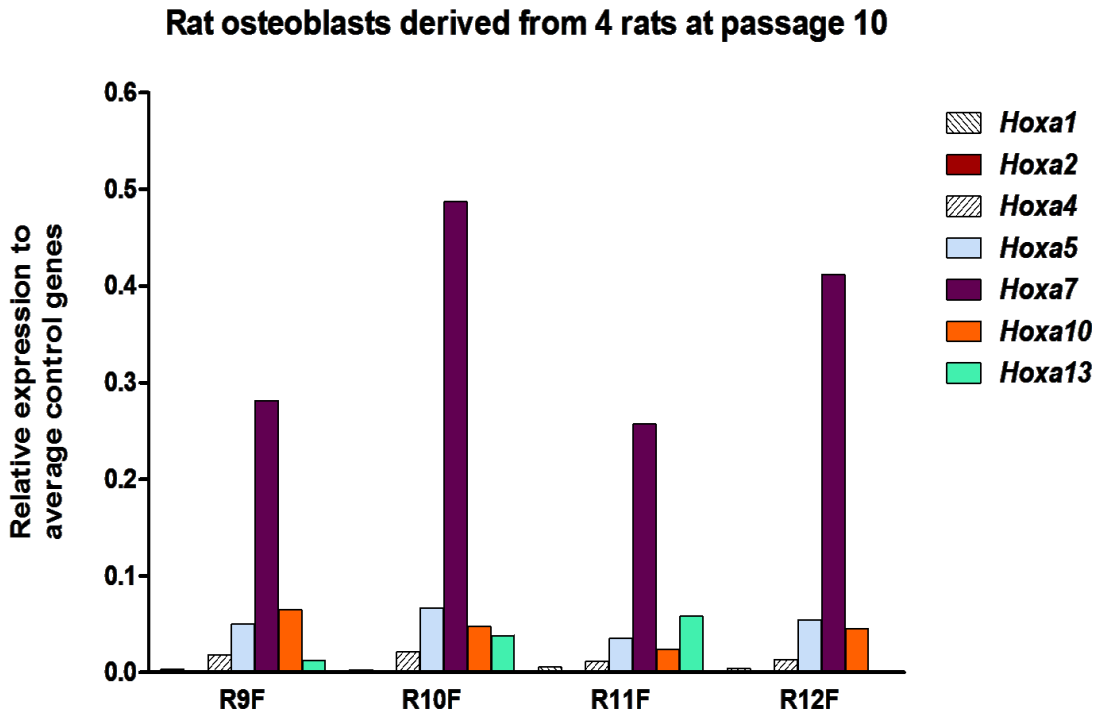


Figure 4-12: Relative expression of *Hoxa* genes to average control genes (*Atp5b* and *Eif4a2*) in adult rat calvarial and femoral osteoblastic cells from 4 male rats (Rats 9-12) matched pairs. Data were generated from using 8 different primary osteoblast cultures and 4 replicates were tested for each culture. There was no *Hoxa* gene expression in calvarial osteoblastic cells (see Appendix 5). This showed pattern of *Hoxa* gene expression at passage 10. *Hoxa7* gene expressed highest in all cultures of femoral osteoblastic cells (R9F= Rat 9 femur, R10F= Rat 10 femur, R11F= Rat 11 femur and R12F= Rat12 femur).

4.4.3.2 Expression of other transcription factors

Calvarial and femoral osteoblastic cells were grown in osteoblast culture medium supplemented with 10 ng/ml of FGF-2. The cells were tested at passage 5 and 10 for expression of reported preferentially expressed transcription factors; *Msx2*, *Irx5* and *Tbx3*.

a) *Msx2* and *Irx5* are preferentially expressed in calvarial osteoblastic cells

Msx2 and *Irx5* were preferentially expressed in calvarial osteoblastic cells both in p5 (Figure 4-13 a and b) and p10 (Figure 4-14 a and b). At passage 5, *Msx2* was expressed at significantly higher levels in calvarial osteoblastic cells than in femoral osteoblastic cells ($p<0.05$) (Figure 4-13 a), whilst at passage 10, *Irx5* was significantly higher expressed in calvarial osteoblastic cells than in femoral osteoblastic cells ($p<0.05$) (Figure 4-14 b).

b) *Tbx3* is preferentially expressed in femoral osteoblastic cells

Tbx3 was preferentially expressed in femoral osteoblastic cells both at p5 (Figure 4-13 c) and p10 (Figure 4-14 c). At passage 5, *Tbx3* expression was higher in femoral osteoblastic cells than in calvarial osteoblastic cells, but this did not reach statistical significance ($p<0.05$) (Figure 4-13 c). At passage 10, *Tbx3* was significantly higher expressed in femoral osteoblastic cells than in calvarial osteoblastic cells ($p<0.05$) (Figure 4-14 c).

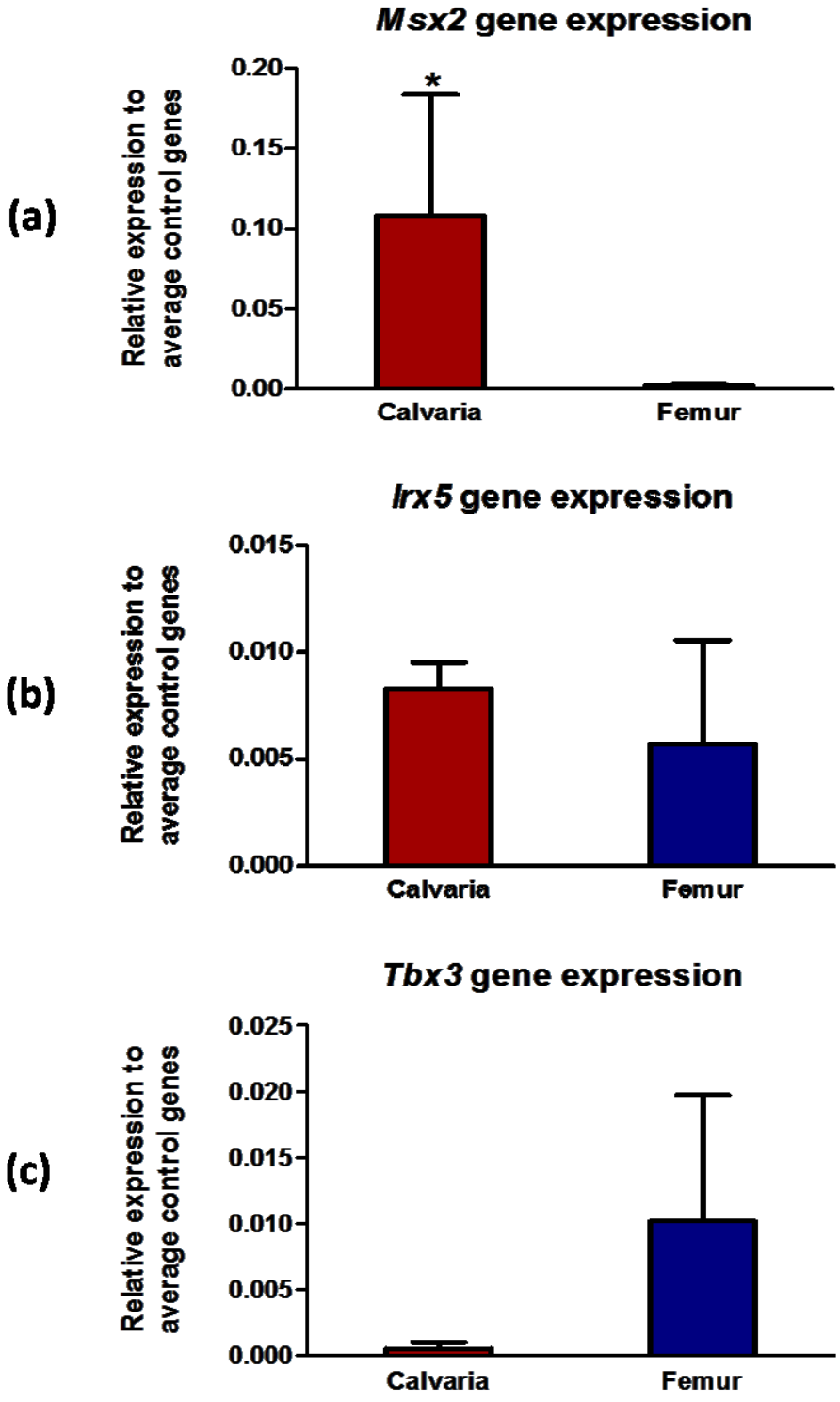


Figure 4-13: Relative expression of transcription factors to average control genes (*Atp5b* and *Eif4a2*) in adult rat calvarial and femoral osteoblastic cells at passage 5. Data represented as mean ± SD from 4 different male rats matched pairs. Data were generated from using 8 different primary osteoblast cultures and 4 replicates were tested for each culture. This showed *Msx2* and *Irx5* genes preferentially expressed in calvarial osteoblastic cells, whilst *Tbx3* preferentially expressed in femoral osteoblastic cells. (a) *Msx2* expressed significantly higher in calvarial osteoblastic cells (*, $p<0.05$), (b) *Irx5* also expressed higher in calvarial osteoblastic cells, but no significantly different ($p<0.05$), whilst (c) *Tbx3* expressed higher in femoral osteoblastic cells, but no significantly different ($p<0.05$). Paired *t*-test was used to compare gene expression between adult rat calvarial and femoral osteoblastic cells (*, $p<0.05$ was considered significant).

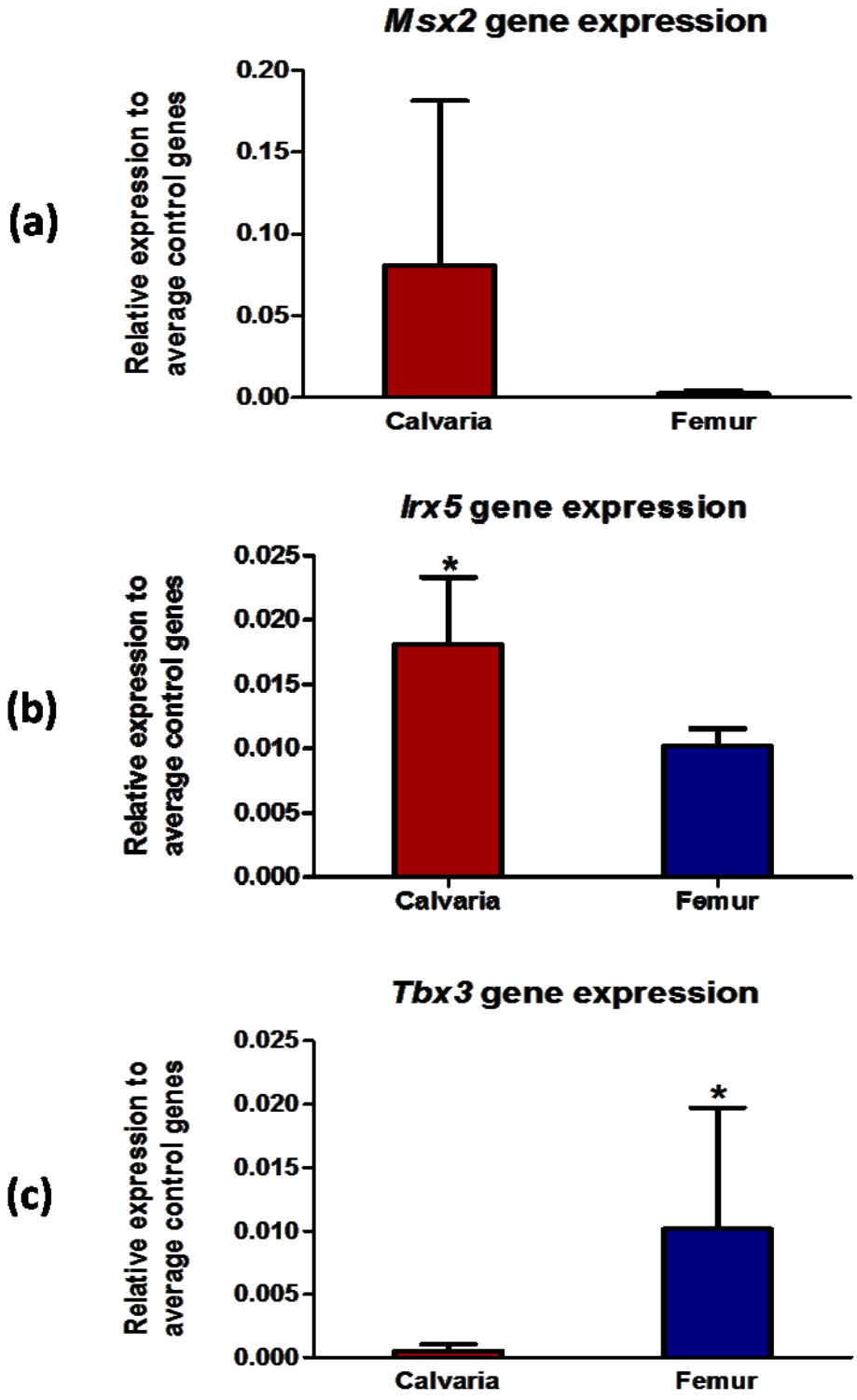


Figure 4-14: Relative expression of transcription factors to average control genes (*Atp5b* and *Eif4a2*) in adult rat calvarial and femoral osteoblastic cells at passage 10. Data represented as mean ± SD from 4 different male rats matched pairs. Data were generated from using 8 different primary osteoblast cultures and 4 replicates were tested for each culture. This showed *Msx2* and *Irx5* genes preferentially expressed in calvarial osteoblastic cells, whilst *Tbx3* preferentially expressed in femoral osteoblastic cells. (a) *Msx2* expressed higher in calvarial osteoblastic cells, but no significantly different ($p<0.05$), (b) *Irx5* expressed significantly higher in calvarial osteoblastic cells (*, $p<0.05$), whilst (c) *Tbx3* expressed significantly higher in femoral osteoblastic cells (*, $p<0.05$). Paired *t*-test was used to compare gene expression between adult rat calvarial and femoral osteoblastic cells (*, $p<0.05$ was considered significant).

4.4.3.3 Additional analyses of transcription factor expression

Although a number of the predicted differences in transcription factor expression did not show significant differences, the "direction" of the difference in expression of specific factors between femoral and calvarial cells appeared remarkably consistent. In order to explore the possibility that lack of statistical significance was the result in the large standard deviations of data inherent in testing a number of different cells, further analyses were carried out pooling the data from p5 and p10 for each culture, and also including additional data obtained from monotypic cultures in further experiments described in Chapter 5. This was considered justifiable as there were no consistent differences in overall expression patterns from different experiments. Consequently, there were data from a total of 8 different experiments for these analyses. Data were all normalised to describe expression patterns of femoral cells as a percentage of that for calvarial cells and results for cells derived from each animal were analysed separately. Differences between femoral and calvarial cells were then analysed by Wilcoxon's Sign-Rank test.

In these additional analyses, *Msx2* and *Irx5* expression were significantly higher in calvarial osteoblastic cells than in femoral osteoblastic cells (Figures 4-15 and 4-16) in 3 of 4 rats, whilst *Tbx3* expression was significantly higher in femoral osteoblastic cells than in calvarial osteoblastic cells (Figure 4-17) in all 4 rats.

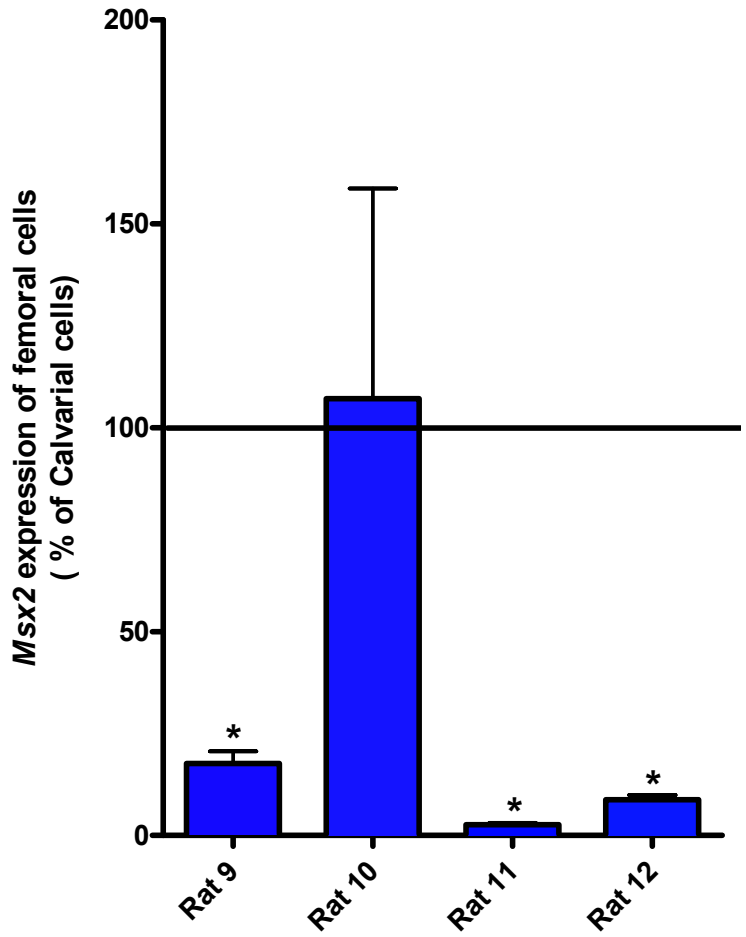


Figure 4-15: Relative expression of *Msx2* to average control genes (*Atp5b* and *Eif4a2*) of femoral osteoblastic cells corrected to % of calvarial osteoblastic cells. Data represented as mean ± SD from 8 independent experiments for each rat matched pairs. Data were generated from using 8 different primary osteoblast cultures and 4 replicates were tested for each culture. This showed *Msx2* expressed significantly higher in calvarial osteoblastic cells than in femoral osteoblastic cells. Wilcoxon's Sign-Rank test was used to compare gene expression between adult rat calvarial and femoral osteoblastic cells (*, $p<0.05$ was considered significant).

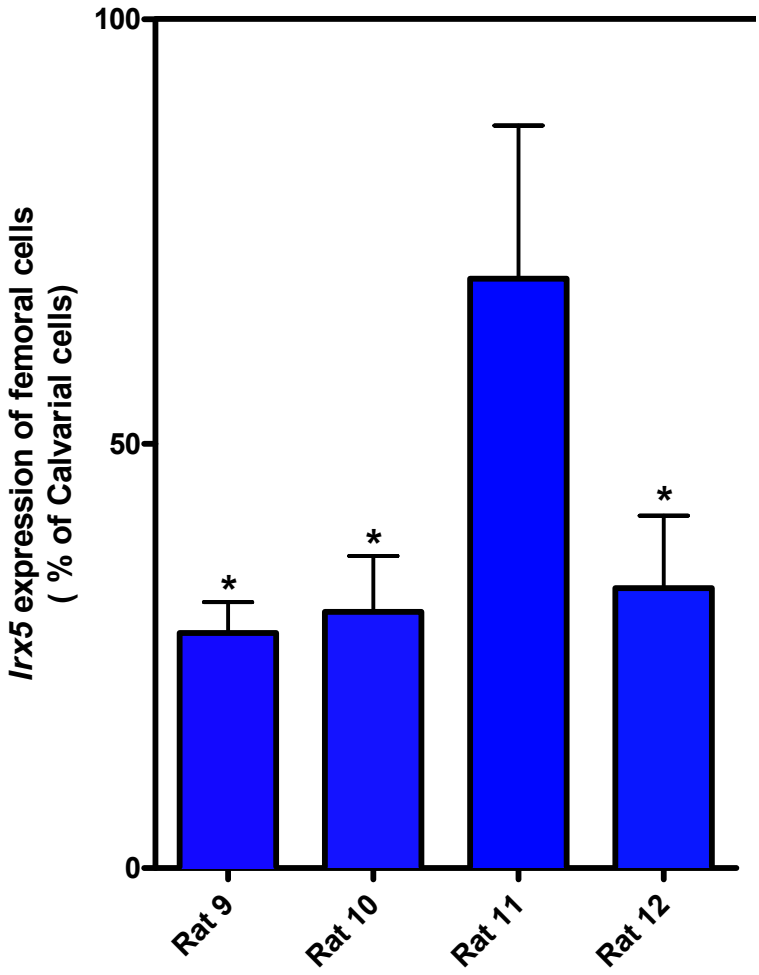


Figure 4-16: Relative expression of *Irx5* to average control genes (*Atp5b* and *Eif4a2*) of femoral osteoblastic cells corrected to % of calvarial osteoblastic cells. Data represented as mean ± SD from 8 independent experiments for each rat matched pairs. Data were generated from using 8 different primary osteoblast cultures and 4 replicates were tested for each culture. This showed *Irx5* expressed significantly higher in calvarial osteoblastic cells than in femoral osteoblastic cells. Wilcoxon's Sign-Rank test was used to compare gene expression between adult rat calvarial and femoral osteoblastic cells (*, $p<0.05$ was considered significant).

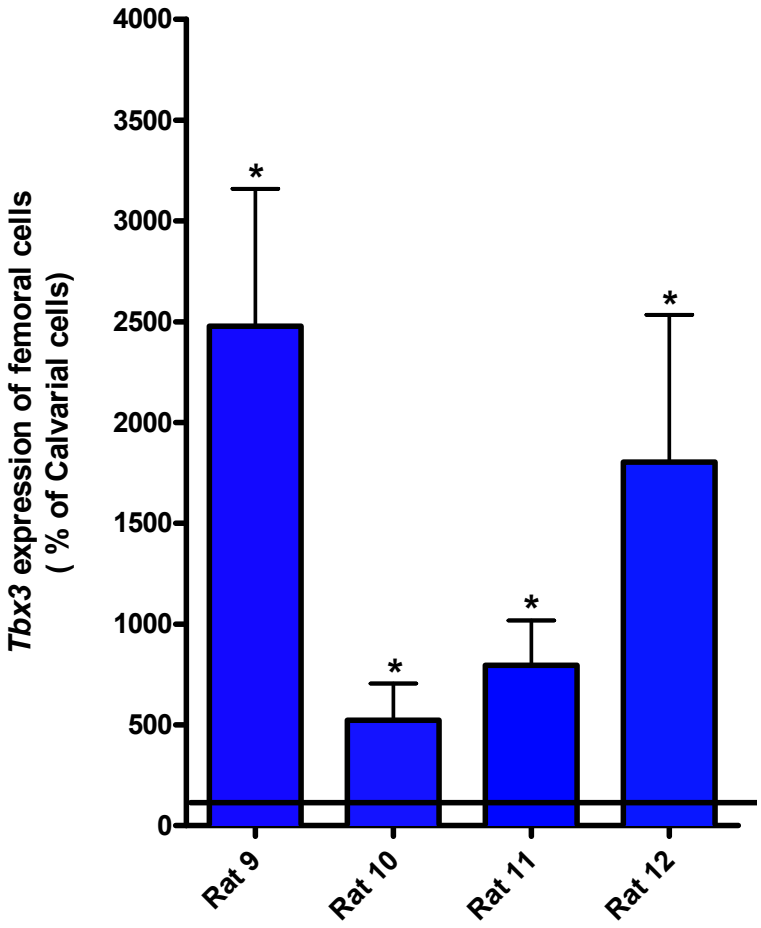


Figure 4-17: Relative expression of *Tbx3* to average control genes (*Atp5b* and *Eif4a2*) of femoral osteoblastic cells corrected to % of calvarial osteoblastic cells. Data represented as mean ± SD from 8 independent experiments for each rat matched pairs. Data were generated from using 8 different primary osteoblast cultures and 4 replicates were tested for each culture. This showed *Tbx3* expressed significantly higher in femoral osteoblastic cells than in calvarial osteoblastic cells. Wilcoxon's Sign-Rank test was used to compare gene expression between adult rat calvarial and femoral osteoblastic cells (*, $p<0.05$ was considered significant).

4.5 Discussion and conclusion

In agreement with previous findings (Rinn et al., 2008b), dermal fibroblasts showed patterns of *Hox* expression consistent with their regional origins. Fibroblasts are differentiated based on their position along three anatomic divisions; anterior-posterior, proximal-distal, and dermal-nondermal (Rinn et al., 2006). The "*HOX* code" may dictate the positional identity of skin and influence site-specific epidermal differentiation (Chuong, 1993).

The results here demonstrated that adult rat fibroblasts showed persistence of embryonically programmed positional identity, as defined by *Hoxa* expression, in cell cultures. *Hoxa* gene expression profiles persisted in fibroblasts consistent with their embryonic expression patterns. There was no *Hoxa* expression seen in fibroblasts from the scalp. In contrast, cells derived from distal forelimb region mainly express *Hoxa7* and *Hoxa10*, from abdomen *Hoxa2*, *Hoxa4*, *Hoxa5* and *Hoxa13* and from shoulder region *Hoxa1*. Persistence of expression was stable for at least 5 subcultures.

The rat dermal fibroblasts derived from different embryonic origins such as head connective tissues derived from ectoderm germ layers, whilst other connective tissues are derived from mesoderm germ layers, have also shown different positional identity in different sites in culture. Adult rat connective tissues have been also shown to retain defined patterns of gene expression as part of the legacy of their differentiation.

On the other hand, although the BMSCs here (femur, humerus, tibia, ulna) are derived from lateral plate mesoderm, we found that they retained their distinct positional identity markers even though they are derived from the same embryonic origin, except ribs which are derived from somites and paraxial mesoderm. We identified *Hoxa* gene expression and gene expression pattern in BMSCs obtained from the marrow from distinct bony regions that are consistently expressed in all BMSCs primary cultures. Particularly, BMSCs obtained from the marrow from ribs showed a distinct expression pattern with greatest expression of *Hoxa5*, instead of *Hoxa7* like other regions. However, *Alp*, *Osteocalcin* and *Tgfbi* genes were not differently expressed in BMSCs primary cultures established from the marrow obtained from distinct bony regions.

Bone marrow is a complex tissue containing stem cells which reside in the marrow cavity and provide support for haematopoiesis (Dexter and Spooncer, 1987). BMSCs are phenotypically and functionally heterogeneous (Dexter et al., 1977). They are fibroblastic in appearance, may have self-renewal potential and can differentiate into several cell lineages including osteoblasts, chondrocytes, adipocytes, myelosupportive stroma, and myoblasts (Prockop, 1997). BMSC cultures have often been used as a source of osteoblastic cells in culture, as they contain both endosteal osteoblasts and osteoprogenitor cells, and less differentiated cells capable of undergoing osteoblastic potential. Significant differences in the pattern of gene expression in BMSC cultures were seen here.

The pattern of *Hoxa* gene expression in the bone marrow largely matches the pattern of expression seen in osteoblast cultures isolated from these bones (Rawlinson et al., 2009b). Clearly, it will be important to understand if the expression seen in cultured bone marrow is restricted to cells of the osteoblast lineage or is representative of the entire population of the bone marrow stromal cells. Thus bone marrow cells appear to show a positional identity which is related to the positional identity of their adjacent bone. The question then arises as to whether this positional identity will persist in the event of cell migration and colonisation to sites distant from their site of origin.

BMSCs that populate the medullary cavities of bones are able to contribute to the maintenance and repair of many tissues. The possibility that the bone marrow cells retain regional identity raises interesting questions regarding the stability and effect of this identity when marrow cells colonise sites distant to their site of origin.

FGF signalling preferentially regulates the 5' (more-posterior) *Hox* genes, whereas RA signalling preferentially regulates the 3' (more-anterior) *Hox* genes (Bel-Vialar et al., 2002). FGF signalling in mouse and chick embryos during posterior body axis extension have shown that FGF signalling is high and can maintain an undifferentiated state at the posterior end of the axis (Dorey and Amaya, 2010). FGF may stimulate expression of *Hoxa7*. Based on our data, the *Hoxa* gene expression pattern in calvarial and femoral osteoblastic cells obtained from different rats was notably consistent throughout the time in culture. *Hoxa* gene expression was maintained even in the present of FGF-2.

Significant differences of *Hoxa* gene expression between regionally distinct populations were seen. There was no *Hoxa* gene expression seen in calvarial osteoblastic cells, which are of paraxial mesodermal-derived origin, whilst femoral osteoblasts cells, which are of lateral plate mesoderm-derived embryonic origin expressed the pattern of *Hoxa* gene expression throughout which matches the pattern of expression seen in osteoblast cultures isolated from these bones (Rawlinson et al., 2009b). One of the key questions to be addressed by this experiment is whether this "positional identity" would persist with long term culture.

From our data, the positionally determined expression of *Hoxa* genes persisted in adult cells and was maintained in cells in culture by the cells themselves may be due to cell memory, not because of the environment such as grown in FGF-2 supplemented in culture media which shown its useful for proliferation, but not differentiation. At passage 10 there was no sign of even a decline in *Hoxa* gene expression and indeed although the expression data obtained at passage 5 and passage 10 may not be strictly comparable quantitatively, relative *Hoxa* expression was seen to be higher at passage 10 than at passage 5 suggesting that the more mature the primary osteoblast cultures, the more stable of the pattern among individual rats and reaffirm the positional information.

Interestingly, *Hoxa13* was expressed in primary fibroblast cultures derived from dermal tissue overlying the abdominal region, whilst *Hoxa13* was expressed in primary osteoblast cultures derived from ulnae and femurs. *Hoxa13* expression was low in ribs compare to ulna. In fact, *Hoxa13* should be expressed in the more posterior region. This may suggest that positional identity is more rigid for skeletal body patterning, whilst it is less fixed in retaining positional information in skin body patterning. Nevertheless, *Hoxa13* expression was maintained and the embryonic *Hox* expression pattern persisted to adulthood, and somewhat followed colinearity.

For transcription factors assessed by qRT-PCR, we have found that *Msx2* and *Irx5* genes were preferentially expressed in calvarial osteoblastic cells. *Msx2* expression was significantly higher at passage 5, whilst *Irx5* was significantly higher at passage 10. Interestingly, *Tbx3* gene was preferentially expressed in femoral osteoblastic cells at passage 10. Based on our data, less mature osteoblastic cells at p5 showed similar *Tbx-3* expression between calvariae and fumurs, whilst more mature osteoblastic cells at p10

showed different *Tbx-3* expression between calvariae and fumurs, close to a statistically significant difference. In addition, the more mature calvarial osteoblastic cells at p10, the more difference in *Msx2* and *Irx5* expression. The variation among inbred rats in gene expression profiles may be due to epigenetic factors and may have an impact on the *Hoxa* genes result outcome. Therefore, we reanalysed our data using 8 independent experiments for each matched pair of rat cell cultures. The results have confirmed that *Msx2* and *Irx5* were significantly higher expressed in calvarial osteoblastic cells, whilst *Tbx3* was significantly expressed higher in femoral osteoblastic cells.

These results suggest that osteoblasts derived from distinct sites are phenotypically different and may show important functional differences. We could demonstrate the principle of positional identity in adult rat dermal fibroblasts, BMSCs, calvarial and femoral osteoblastic cells.

Overall the results of the experiments described here suggest that expression of regionally distinct positional identity markers such as *Hoxa* may be the result of inherent cellular programming rather than being related to environmental cues. Based on our data, *Hoxa* gene expression was maintained even in the present of FGF-2. Furthermore, these markers may be more a reflection of their anatomic location in the developing organism rather than being related specifically to their embryonic origin. More favourable clinical outcomes in bone grafting and tissue regeneration are expected with site matching between donor and recipient.

In conclusion, adult rat dermal fibroblasts, BMSCs and primary osteoblast cultures derived from different embryonic origins showed persistence and distinct patterns of embryonically programmed positional identity in a site-specific manner. FGF-2 did not interfere with *Hoxa* gene expression in adult rat primary osteoblast cultures as shown by persistence of positional identity in culture up to 10 subcultures. The positional identity appeared to be more rigid for skeletal body patterning, whilst it was less stable in retaining positional information in skin body patterning. The less mature osteoblastic cells showed similar *Tbx-3* expression, whilst more mature osteoblastic cells showed differences in *Tbx-3* expression between calvariae and fumurs. In addition, the more mature the calvarial osteoblastic cells were, the greater the difference in *Msx2* and *Irx5* expression was observed.

Chapter 5

Chapter 5: Modulation of positional identity

5.1 Introduction

In Chapter 4, we have demonstrated that the positional identity persisted in cultures up to 10 passages. There was a difference between adult rat calvarial osteoblastic cells and femoral osteoblastic cells in *Hoxa* gene expression. The calvarial osteoblastic cells which were derived from paraxial mesoderm maintained their *Hoxa* negative gene expression, whilst lateral plate mesodermal-derived femoral osteoblastic cells showed persistence and pattern of *Hoxa* gene expression *in vitro*.

Leucht and co-workers tested the modulation of positional identity by grafting the cells from different embryonic origin to determine whether they are interchangeable in bone grafting or not (Leucht et al., 2008a). This study tested for wound healing and regenerative potentials by labelling neural crest-derived cells and mesoderm-derived cells, and then produced skeletal injuries in a mandible, which is a neural crest-derived bone, and in a tibia, which is a mesoderm-derived bone. They found that bones heal through cells from their own embryonic origins (Leucht et al., 2008a).

Interestingly, placing neural crest-derived periosteum into a mesoderm-derived injury site resulted in intramembranous bone formation, but placing tibial periosteum into a mandibular injury site resulted in endochondral ossification and transplanted cells underwent hypertrophy even though the environment fully supported osteogenic differentiation (Leucht et al., 2008a).

In keeping with our results their results indicate that stem cells from skeleton have 'positional memory', which influences the cells behavior when grafted into ectopic locations (Leucht et al., 2008a). Therefore, this *in vivo* finding of enhanced osteogenesis in neural crest transplants may be explained by the increasing cell osteogenic potential. The osteoprogenitor cells derived from neural crest formed new bone regardless of whether they were transplanted into a tibial defect or into another mandibular injury site (Leucht et al., 2008a).

The question still remains at the molecular level as to why skeletal progenitor cells of mesoderm origin did not appear to exhibit the same plasticity as skeletal progenitor cells of neural crest origin when they were transplanted into mandibular defects. The

mesodermal cells differentiated into chondrocytes even though grafted in the environment that fully supported osteogenic differentiation. These results suggested that there was a fundamental difference in the plasticity of progenitor cells from two distinct origins and suggest that the local environment may have induced previously Hox-negative cells to become Hox-positive because of the action of local signals.

Given that our results described in Chapter 4 show persistence of positional identity markers even when maintained for long periods of time away from any potential environmental cues, the question arises that can *Hoxa* gene expression change and if so, what induces these changes? These are challenging questions and therefore we tried to answer some of those questions by carrying out the experiments described in this Chapter. We hypothesized that the *Hoxa* gene expression can be modulated in *Hoxa*-negative cells due to soluble factors or local growth factors (Hughes et al., 2006) from *Hoxa*-positive cells or due to cell to cell contact with *Hoxa*-positive cells in heterotypic cultures.

5.2 Aims

1. To investigate whether soluble factors in conditioned medium can regulate expression of *Hoxa* genes;

2. To investigate whether cell to cell contact in heterotypic cultures can modulate expression of *Hoxa* genes.

5.3 Materials and Methods

5.3.1 The effect of soluble factors on *Hoxa* gene expression using a rat fibroblast model

To address whether soluble factors in conditioned medium (CM) have an effect on positional identity or not, this pilot experiment was carried out using CM to treat Hox-ve and Hox+ve rat dermal fibroblasts and then assess *Hoxa* gene expression by qRT-PCR. Briefly, the Hox-ve and Hox+ve rat dermal fibroblasts were grown in T-25 flasks in culture medium. The conditioned media from Hox-ve and Hox+ve rat dermal fibroblasts were collected and added back to the cells. In the first group, Hox-ve or Hox+ve without CM, only normal growth culture medium was added back. They were used as a baseline control. The second group, Hox-ve or Hox+ve with same CM were used to assess the relative effect of the test CM on *Hoxa* gene expression. The third group, the heterotypic Hox cells were treated with different CM such as Hox+ve cells were treated with CM from Hox–ve cells, whilst Hox–ve cells were treated with CM from Hox+ve cells. After 3 days in the CM, *Hoxa* gene expression was assessed by qRT-PCR. This experiment is summarised and depicted in Figure 5-1.

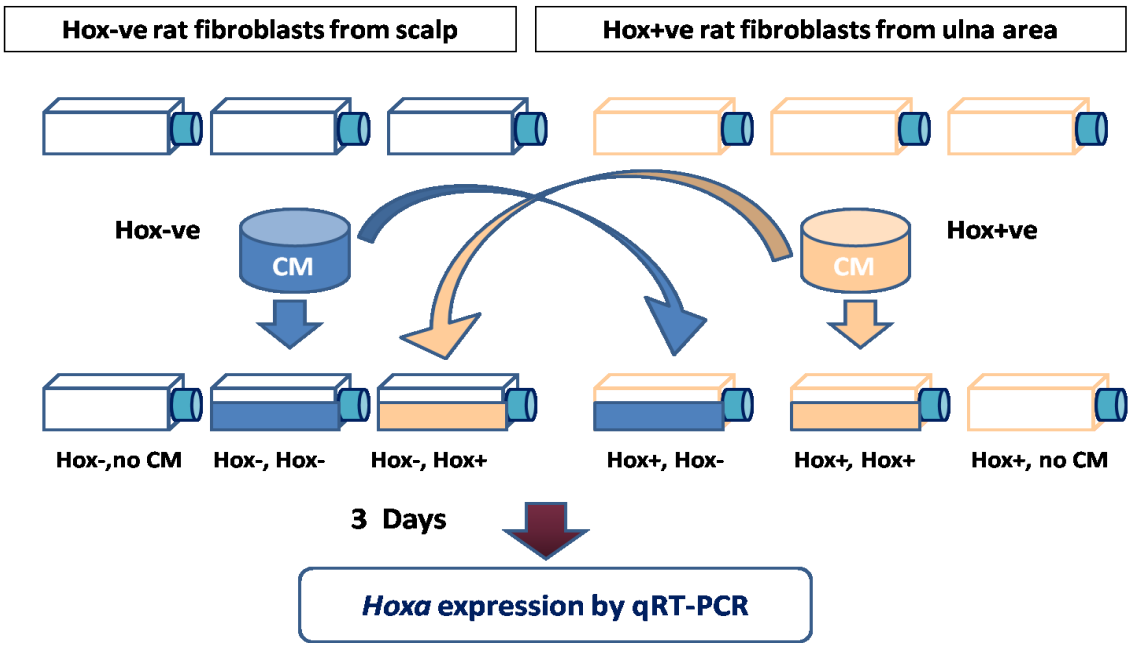


Figure 5-1: Hox-ve and Hox+ve rat dermal fibroblasts were grown separately until reached 80-90% confluence. The conditioned medium (CM) were collected and added back to the cells. Briefly, the first group, Hox-ve or Hox+ve without CM (only normal growth culture medium added back) were used as a baseline control. The second group, Hox-ve or Hox+ve with same CM were used to assess the relative effect of the test CM on *Hoxa* gene expression. The third group, the heterotypic Hox cells were treated with different CM such as Hox+ve cells were treated with Hox–ve CM, whilst Hox–ve cells were treated with Hox+ve CM. After 3 days in the CM mentioned earlier, *Hoxa* gene expression was assessed by qRT-PCR.

5.3.2 Cell labelling

In order to assess the effects of direct cell to cell contacts in heterotypic cultures of *Hoxa*-positive and *Hoxa*-negative cells in modulating positional identity we aimed to pre-label *Hoxa*-positive and negative cells with fluorescent dyes prior to mixing cells in heterotypic cultures. The pre-labelled cell populations would then be sorted by flow cytometry back into their monotypic populations, and any changes in *Hoxa* expression determined. Specifically these experiments test whether *Hoxa*-negative cells may become *Hoxa* positive following direct cell-cell contact in the heterotypic cultures. The cell labelling method has been previously described in section 2.10.

5.3.2.1 Persistence of cell labelling

This pilot experiment was carried out to demonstrate that the live fibroblasts can be stained, maintained and the heterotypic fibroblast cultures can be distinguished under fluorescence microscopy. CellTracker^{TM} Green and CellTracker^{TM} Red were used. The cells were maintained in monotypic or heterotypic culture up to 1 week and visualised under fluorescence microscope as previously described in detail in section 2.10.3.

A similar experiment was carried out to demonstrate that the live osteoblastic cells in heterotypic cultures can be stained, maintained and distinguished under fluorescence microscopy. CellTracker^{TM} Green, CellTracker^{TM} Red and CellTracker^{TM} Orange were used. The cells were maintained in monotypic or heterotypic culture for up to 1 week and visualised under fluorescence microscope as described in details in section 2.10.4.

5.3.3 Investigation of transfer of label from one cell type to the other

Experiments were carried out to test if cross-contamination of labelled cells in heterotypic cultures occurred as followed:

a) Using fluorescence microscope

The two different populations were stained and grown together in heterotypic culture for 0 and 3 days. At each time point, the cells were visualised under fluorescence microscope, and photos were taken as previously described in section 2.10.4.

b) FACS analysis of double stained heterotypic cultures

The cells were stained and grown together for 0 and 3 days. At each time point, both cell types were collected for flow cytometry analysis as described in section 2.11. The unstained cells for both cell types were used for calibration.

c) BD Falcon™ Cell culture inserts system and flow cytometry

The two distinct populations were stained and grown in BD Falcon™ Cell culture inserts system (Figure 5-2) for 0, 3 and 7 days and analysed by Flow cytometry at each time point as described in section 2.11.

Figure 5-2: Falcon insert system composes of BD Falcon™ 6-well Cell Culture Insert Companion Plate and BD Falcon™ Cell culture inserts for 6-well plates, 0.4 µm pores, Transparent PET Membrane. From: (BD product catalog, no. 353502 and 353090).

5.3.4 qRT-PCR assessed for *Hoxa* gene expression in monotypic and heterotypic cultures

The two different populations from 4 rats were stained with CellTracker™ Green and CellTracker™ Red, grown separately in monotypic culture or grown together in heterotypic culture for 0, 3 and 7 days. In heterotypic culture the cells were sorted by cell sorter as described in section 2.11. In monotypic cultures, the cells were stained and passed through the cell sorter, but not sorted according to staining in order to act as a control for the cell processing. The cells were collected at each time point and further tested for *Hoxa* gene expression as previously described.

Because of concerns about the possible leakage of CellTracker™ Red and Orange stains, in later experiments we used only CellTracker™ Green labelling on one of the populations, whilst the other population was unstained. These were carried out for both monotypic culture used as control and heterotypic culture by first staining calvarial osteoblastic cells with CellTracker™ Green, whilst femoral osteoblastic cells were left unstained (Figure 5-3). In further experiments, femoral osteoblastic cells were stained with CellTracker™ Green, whilst calvarial osteoblastic cells were left unstained. The cells were grown separately in monotypic culture or grown together in heterotypic culture for 0, 3, and 7 days prior to sorting and testing for *Hoxa* gene expression.

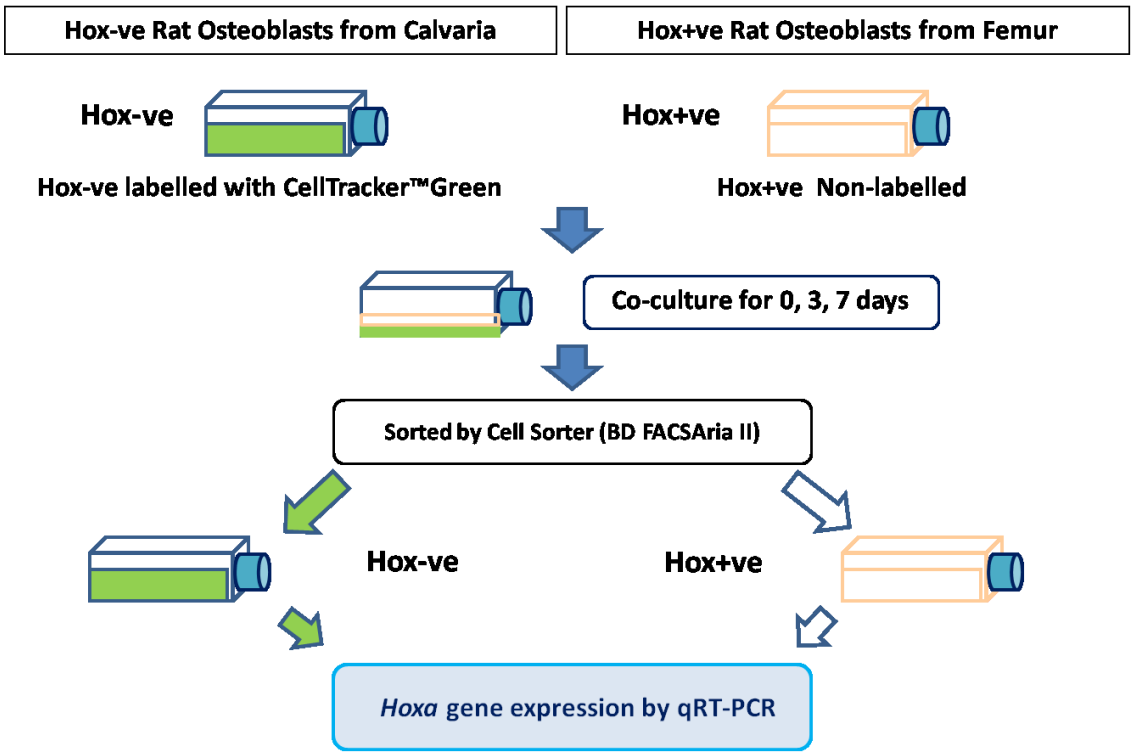


Figure 5-3: Calvarial osteoblastic cells stained with CellTracker™ Green, whilst femoral osteoblastic cells were left unstained. In later experiments, femoral osteoblastic cells were stained with CellTracker™ Green. They were grown separately in monotypic culture or grown together in heterotypic culture for 0, 3 and 7 days. After that, they were passed or sorted by cell sorter and continued to grow until they reached at least 80% confluence and collected for RNA extraction, cDNA synthesis and qRT-PCR to test for *Hoxa* gene expression.

5.4 Results

5.4.1 The effect of soluble factors on *Hoxa* gene expression

As previously demonstrated in Chapter 4, at baseline there was no *Hoxa* gene expression in rat dermal fibroblasts derived from the scalp region. In rat dermal fibroblasts from the ulna area, the pattern of *Hoxa* gene expression can be seen and was maintained throughout (Figure 5-4).

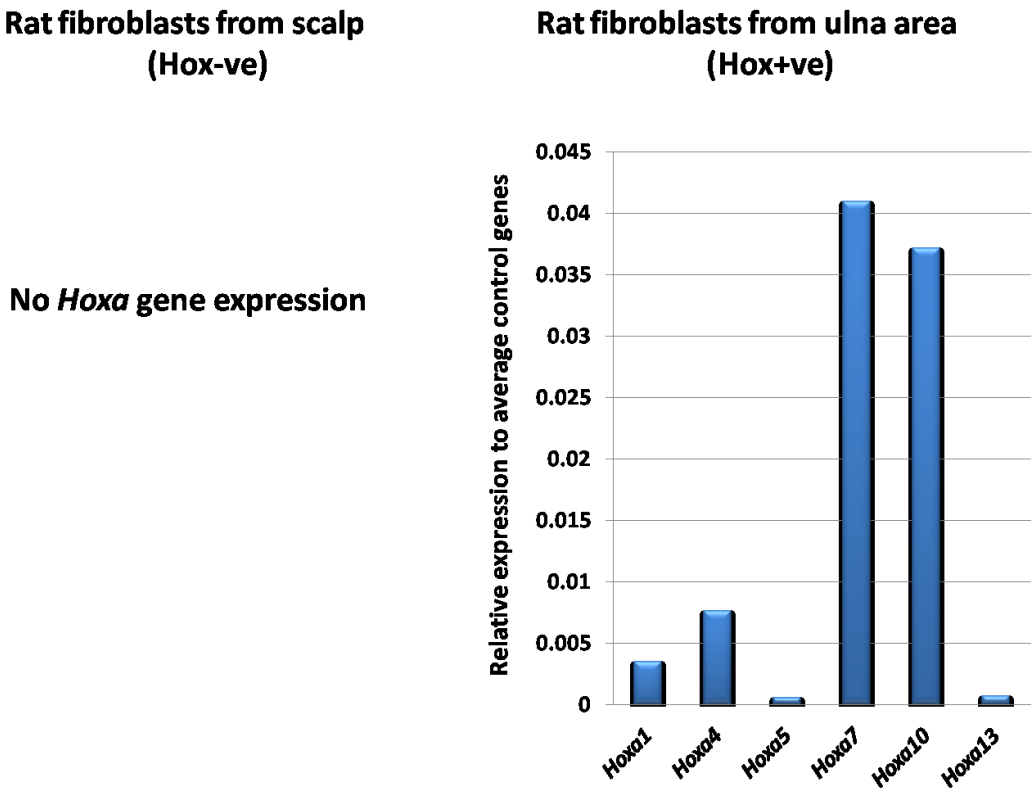


Figure 5-4: There is no *Hoxa* gene expression in rat dermal fibroblasts derived from scalp. In rat dermal fibroblasts from ulna area, there is no *Hoxa2* gene expression, *Hoxa7* was expressed at highest levels, followed by *Hoxa10*. Data were generated from 1 rat.

There was no difference in *Hoxa* expression in ulna dermal fibroblasts when grown in different conditioned medium as shown in Figure 5-5.

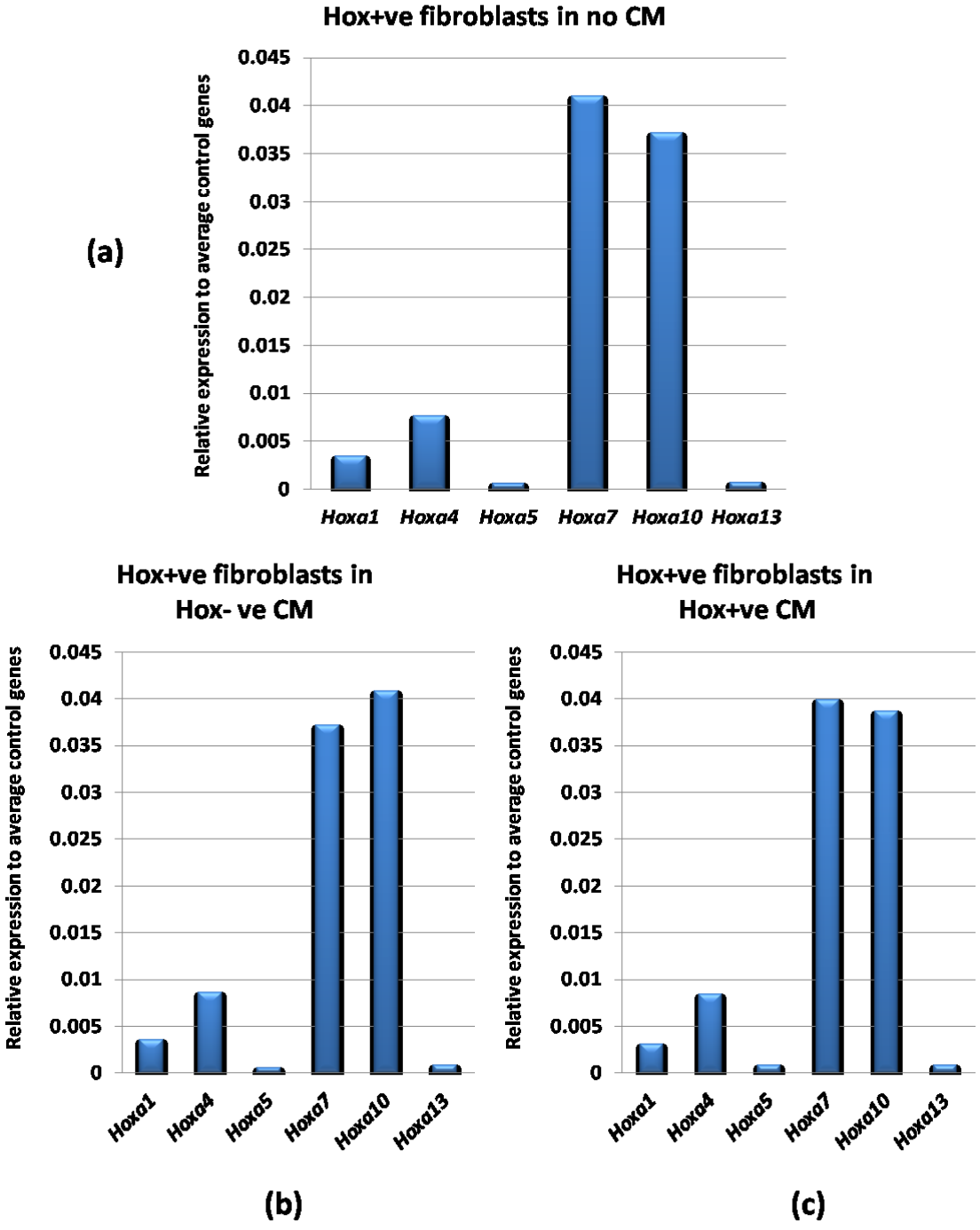


Figure 5-5: After 3 days in conditioned medium (CM), *Hoxa* gene expression was assessed by qRT-PCR; (a) Hox+ve fibroblasts treated with normal growth culture medium used as a baseline control. *Hoxa* gene expression pattern was observed in Hox+ve fibroblasts; (b) Hox+ve fibroblasts treated with Hox-ve CM; (c) Hox+ve fibroblasts treated with Hox+ve CM. There is no effect of conditioned medium on rat dermal fibroblasts *Hoxa* gene expression as similar pattern of *Hoxa* gene expression pattern was evidenced in all conditions. Data were generated from 1 male rat using 2 different rat dermal fibroblasts and 4 replicates were tested for each sample.

Hoxa gene expression was compared between Hox+ve rat dermal fibroblasts treated with Hox-ve CM or Hox+ve CM to control in fold change. This figure shows that there is no difference in the effect of *Hoxa* gene expression in Hox+ve rat dermal fibroblasts treated with different CM during 3 days of experiment. A fold change of 1 indicates no difference from control (Figure 5-6).

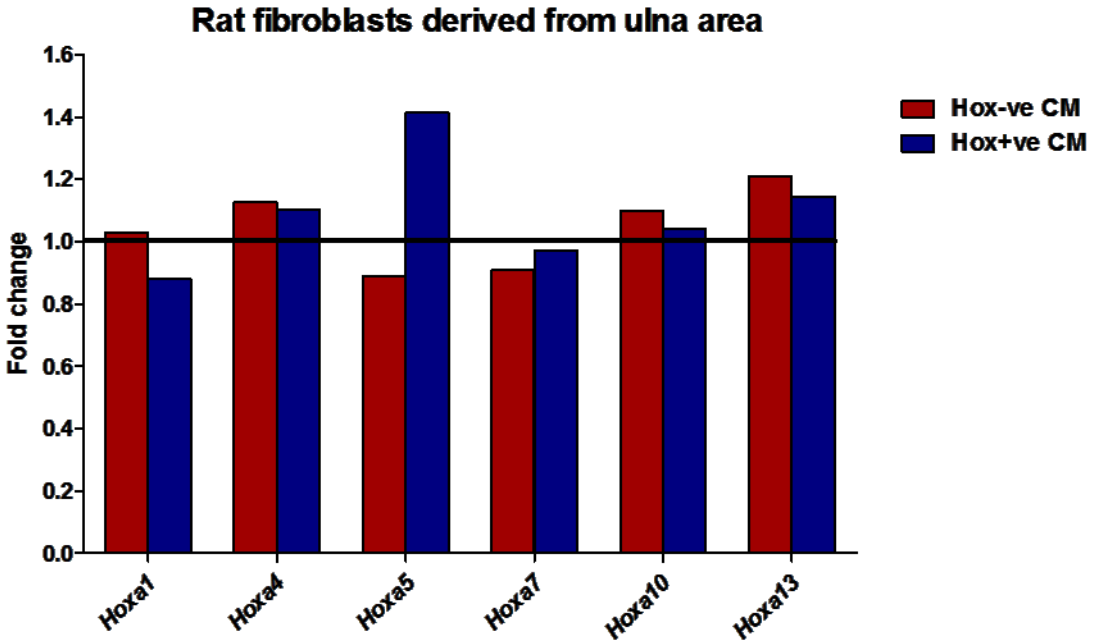


Figure 5-6: *Hoxa* gene expression was compared between Hox+ve rat dermal fibroblasts treated with Hox-ve CM or Hox+ve CM to control in fold change. This figure shows that there is no difference in the effect of *Hoxa* gene expression in Hox+ve rat dermal fibroblasts treated with different CM. A fold change of 1 indicates no difference from control. Data were generated from 1 male rat using 2 different rat dermal fibroblasts and 4 replicates were tested for each sample.

In addition, scalp-derived fibroblasts continued to show no detectable *Hoxa* expression when grown in either Hox-positive CM or negative control medium at least in this time frame.

5.4.2 Persistence of cell labels in fibroblasts

5.4.2.1 Persistence of labelled fibroblasts in monotypic culture

The rat dermal fibroblasts derived from scalp, tested as *Hoxa* negative were stained with CellTracker™ Green. The rat dermal fibroblasts from skin overlying ulna area, tested as *Hoxa* positive were stained with CellTracker™ Red. The cells maintained fluorescence staining up to 7 days and showed normal morphology when looked under fluorescence microscope (Figure 5-7).

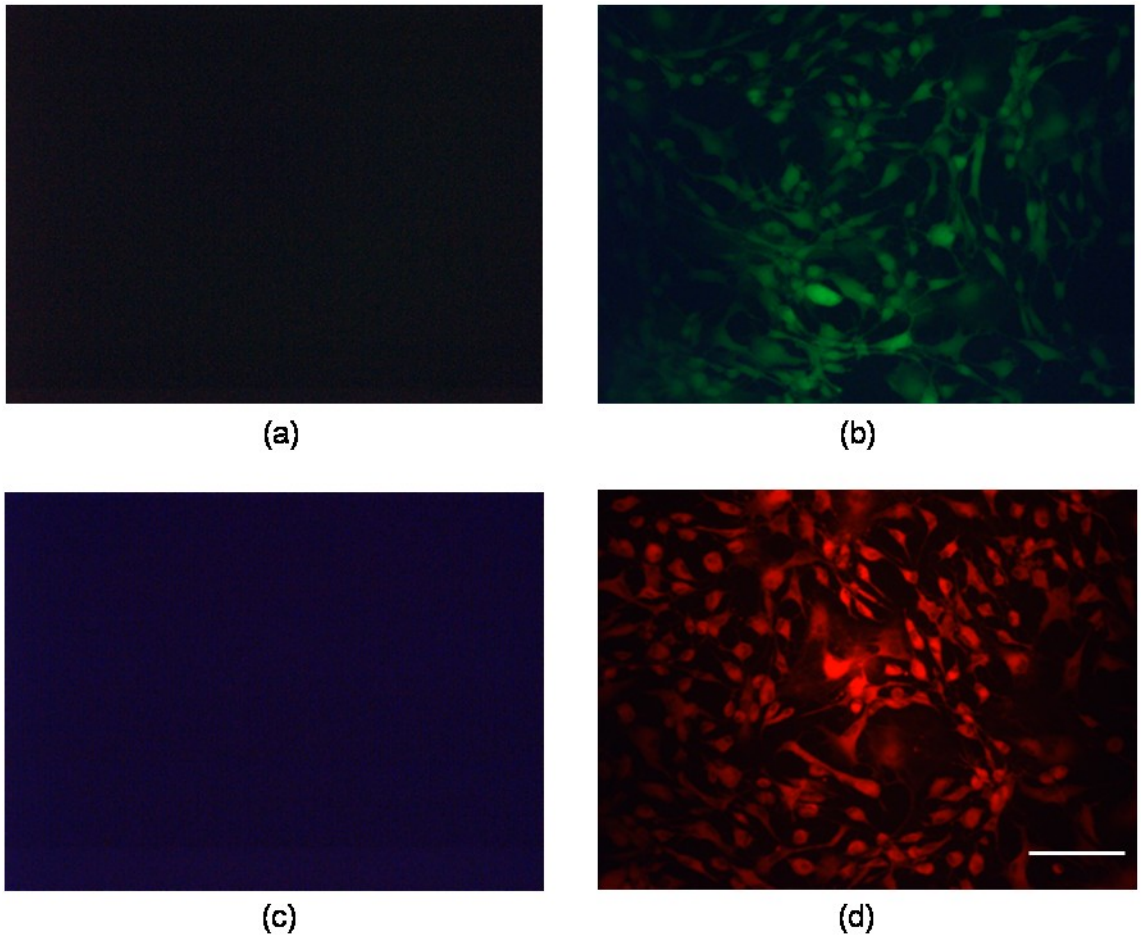


Figure 5-7: (a) Unstained rat dermal fibroblasts from scalp; (b) Rat dermal fibroblasts from scalp stained with CellTracker™ Green; (c) Unstained rat dermal fibroblasts from skin overlying ulna area; (d) Rat dermal fibroblasts from skin overlying ulna area stained with CellTracker™ Red, magnification 200, scale bar = 100μm.

5.4.2.2 Persistence of labelled fibroblasts in heterotypic culture

The rat dermal fibroblasts derived from scalp were stained with CellTracker™ Green, whilst rat dermal fibroblasts from skin overlying ulna area were stained with CellTracker™ Red and grown together in heterotypic culture. The two distinct populations can be distinguished when examined under fluorescence microscope. The photo was taken one colour at the time (Figure 5-8 a and b), and then merged using Photoshop software programme (Figure 5-9).

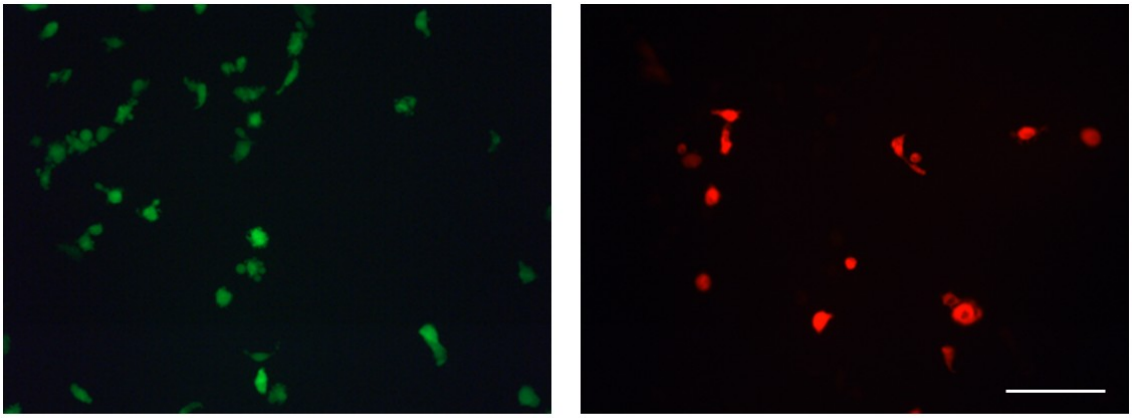


Figure 5-8: Rat dermal fibroblasts derived from; (a) scalp stained with CellTracker™ Green; (b) tissue overlying ulna area stained with CellTracker™ Red at Day 0 at magnification 200, scale bar = 100µm.

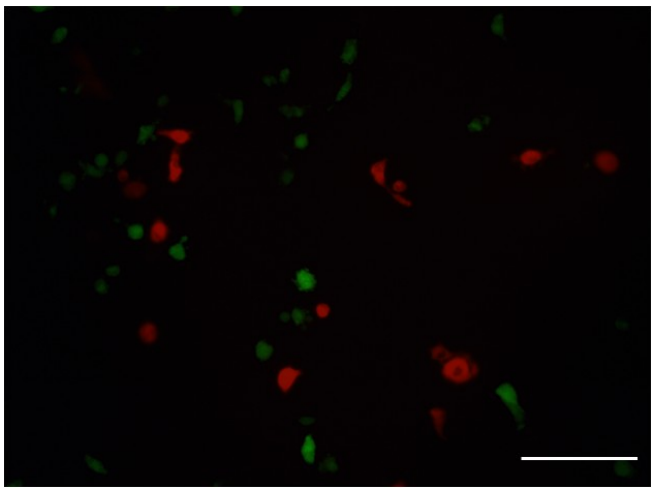


Figure 5-9: Rat dermal fibroblasts derived from scalp stained with CellTracker™ Green and rat dermal fibroblasts derived from tissue overlying ulna area stained with CellTracker™ Red can be distinguished in mixed population at Day 0 in heterotypic culture observed under fluorescence microscope at magnification 200, scale bar = 100µm.

5.4.3 Persistence of cell labels in osteoblast cultures

5.4.3.1 Persistence of labelled osteoblasts in monotypic culture

1) Green/Red

Rat calvarial osteoblastic cells stained with CellTracker™ Green (Figure 5-10 a); rat femoral osteoblastic cells stained with CellTracker™ Red (Figure 5-10 b) observed under fluorescence microscope. The cells can maintain their staining up to 7 days.

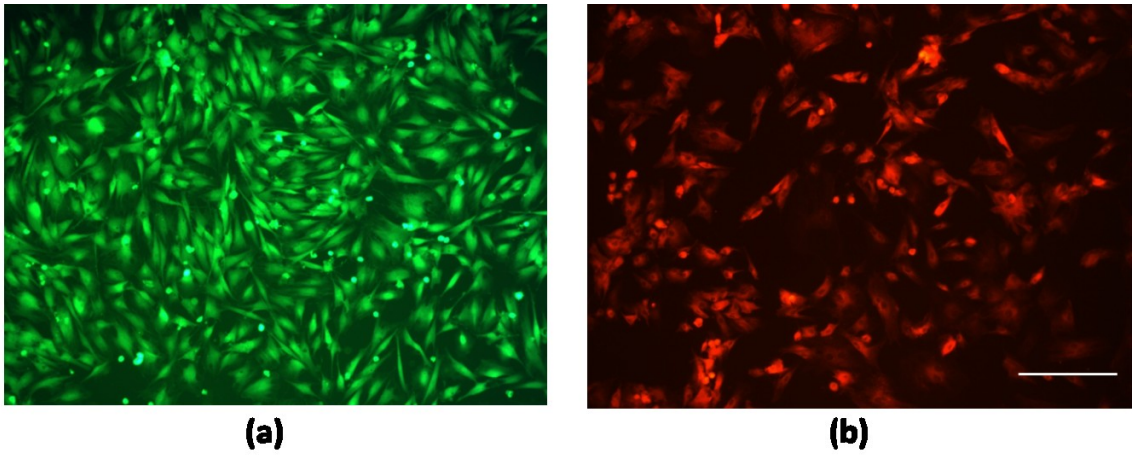


Figure 5-10: (a) Rat calvarial osteoblastic cells stained with CellTracker™ Green; (b) rat femoral osteoblastic cells stained with CellTracker™ Red in monotypic culture observed under fluorescence microscope at Day 3, magnification 100, scale bar = 200μm.

2) Green/Orange

Rat calvarial osteoblastic cells stained with CellTracker™ Green (Figure 5-11 a); rat femoral osteoblastic cells stained with CellTracker™ Orange (Figure 5-11 b) were observed under fluorescence microscope. The cells also can retain their staining up to 7 days.

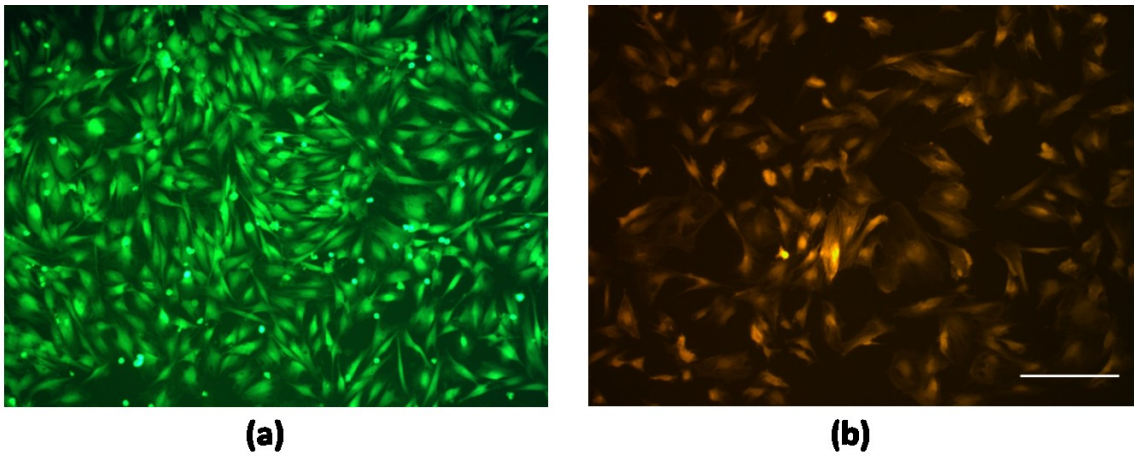


Figure 5-11: (a) Rat calvarial osteoblastic cells stained with CellTracker™ Green; (b) rat femoral osteoblastic cells stained with CellTracker™ Orange in monotypic culture observed under fluorescence microscope at Day 3, magnification 100, scale bar = 200μm.

5.4.3.2 Persistence of labelled osteoblasts in heterotypic culture

1) Green/Red

Rat calvarial osteoblastic cells stained with CellTracker™ Green (Figure 5-12 a); rat femoral osteoblastic cells stained with CellTracker™ Red (Figure 5-12 b); rat calvarial and femoral osteoblastic cells stained with DAPI (Figure 5-12 c); mixed populations of rat calvarial and femoral osteoblastic cells (Figure 5-12 d) observed under fluorescence microscope. Two populations can be clearly identified at Day 0. The photo was taken one colour at a time and then merged using Photoshop software programme (Figure 5-12 d).

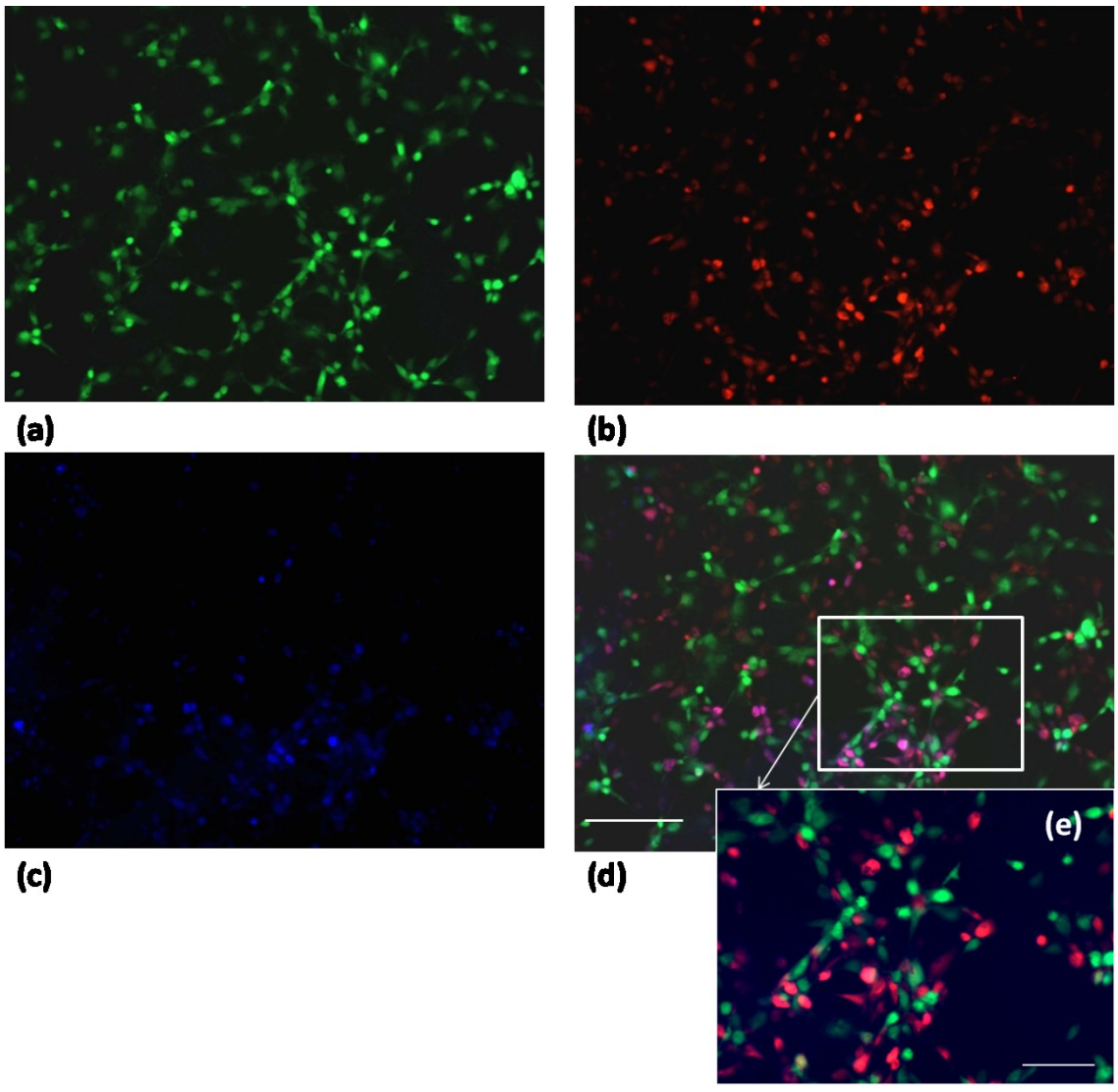


Figure 5-12: (a) Rat calvarial osteoblastic cells stained with CellTracker™ Green; (b) rat femoral osteoblastic cells stained with CellTracker™ Red; (c) rat calvarial and femoral osteoblastic cells stained with DAPI; (d) mixed populations of rat calvarial and femoral osteoblastic cells in heterotypic culture observed under fluorescence microscope at Day 0, magnification 40, scale bar = 100μm; (e) magnification 200, scale bar = 100μm.

However, at Day 3 in heterotypic culture, double staining can be observed (Figure 5-13). Double labelled cells increased with time in culture. Overall, the label was sufficiently retained for 1 week in both green and red dyes (data not shown).

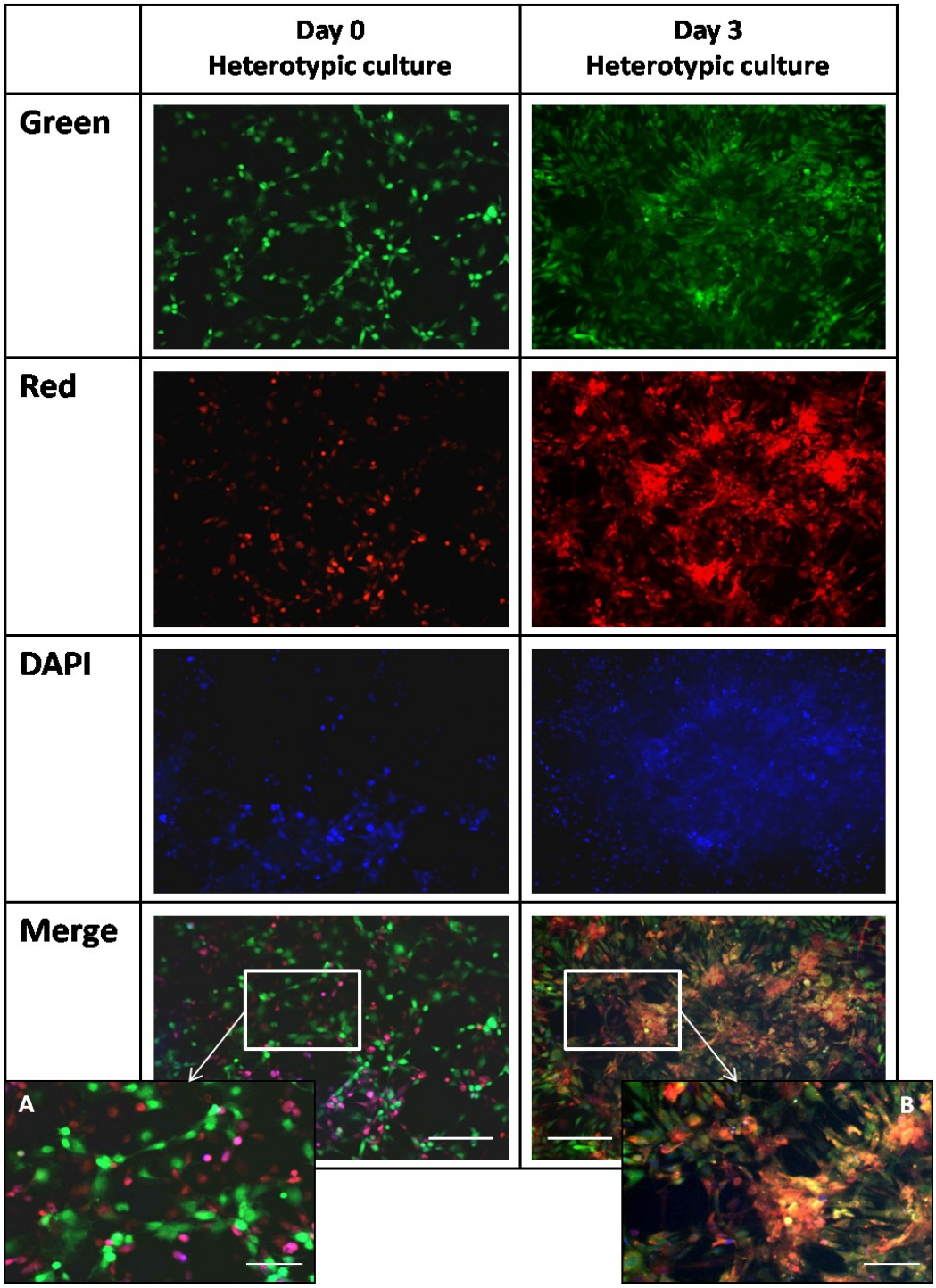


Figure 5-13: Rat calvarial osteoblastic cells stained with CellTracker™ Green; rat femoral osteoblastic cells stained with CellTracker™ Red; rat calvarial and femoral osteoblastic cells stained with DAPI; mixed populations of rat calvarial and femoral osteoblastic cells in heterotypic culture at Day 0 and Day 3 observed under fluorescence microscope. At Day 3, the double staining (orange cells) can be observed, magnification 100, scale bar = 200μm. A and B, magnification 200, scale bar = 100μm.

5.4.4 Evidence of changes of Hox status in osteoblasts following heterotypic cultures

5.4.4.1 Pilot experiment suggest modulation

From our previous data, CellTracker™ Green was the most stable dye. Therefore we carried out these experiments using CellTracker™ Green only to stain our osteoblastic cells from 3 male rats, Rat 9, Rat 11 and Rat 12 as previously described in Figure 5-3.

Surprisingly, *Hoxa* gene expression in calvarial osteoblastic cells was switched on when two distinct Hox populations were grown together in heterotypic culture. *Hoxa* gene expression pattern was observed in calvarial osteoblastic cells at Day 7 in heterotypic culture, similar to *Hoxa* gene expression pattern that expressed in femoral osteoblastic cells (Figures 5-14 and 5-15).

Similar results were also evidenced when tested using osteoblastic cells derived from Rat 11 and Rat 12. In Rat 11, *Hoxa7* was expressed highest, followed by *Hoxa10* and *Hoxa13* (Figure 5-16 and 5-17), whereas in Rat 12 *Hoxa10* was expressed highest, followed by *Hoxa7* and *Hoxa5*, but no *Hoxa13* expression (Figure 5-18 and 5-19).

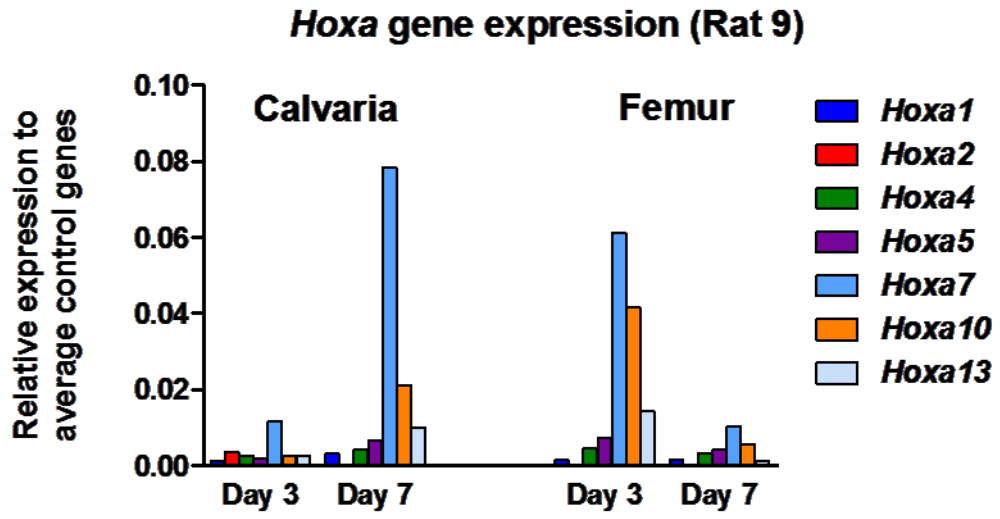


Figure 5-14: Data from Rat 9 in heterotypic culture. The calvarial osteoblastic cells were stained green, whilst femoral osteoblastic cells were unstained. *Vice versa*, calvarial osteoblastic cells were unstained, whilst femoral osteoblastic cells were stained green. Then cells stained cells were sorted for RNA extraction, cDNA synthesis and qRT-PCR. *Hoxa* gene expression was switched on in calvarial osteoblastic cells. At Day 7 in heterotypic culture, *Hoxa* gene expression pattern was observed. *Hoxa7* expressed highest, followed by *Hoxa10* and *Hoxa13*. Data were generated from using 2 different primary osteoblast cultures and 4 replicates were tested for each culture.

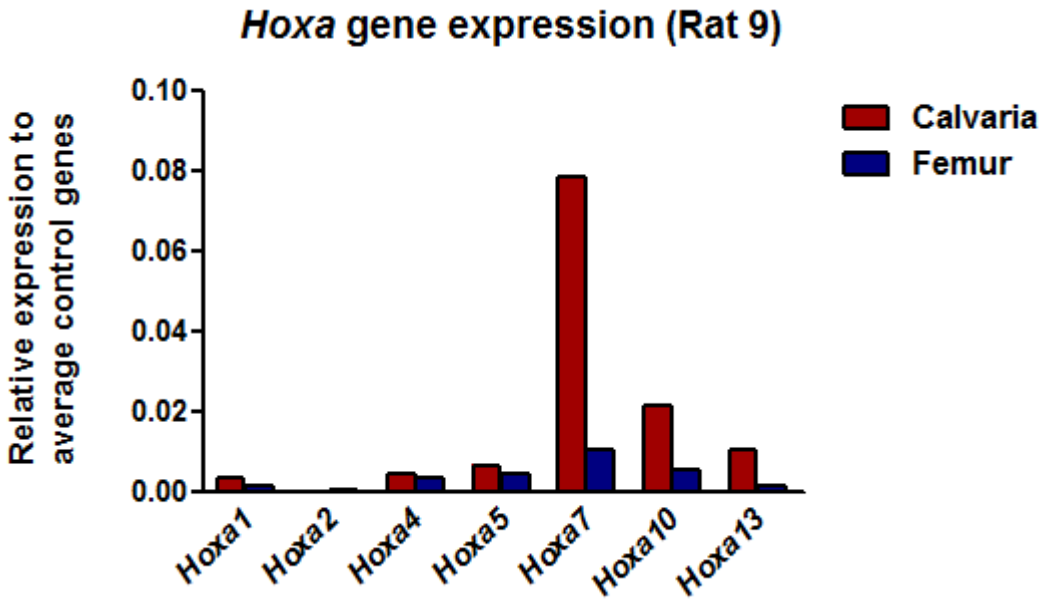


Figure 5-15: Data from Rat 9 calvarial osteoblastic cells were stained green in heterotypic culture when looked closely at Day 7, *Hoxa* gene expression was switched on in calvarial osteoblastic cells. *Hoxa* gene expression pattern was observed. *Hoxa7* expressed highest, followed by *Hoxa10* and *Hoxa13*. Data were generated from using 2 different primary osteoblast cultures and 4 replicates were tested for each culture.

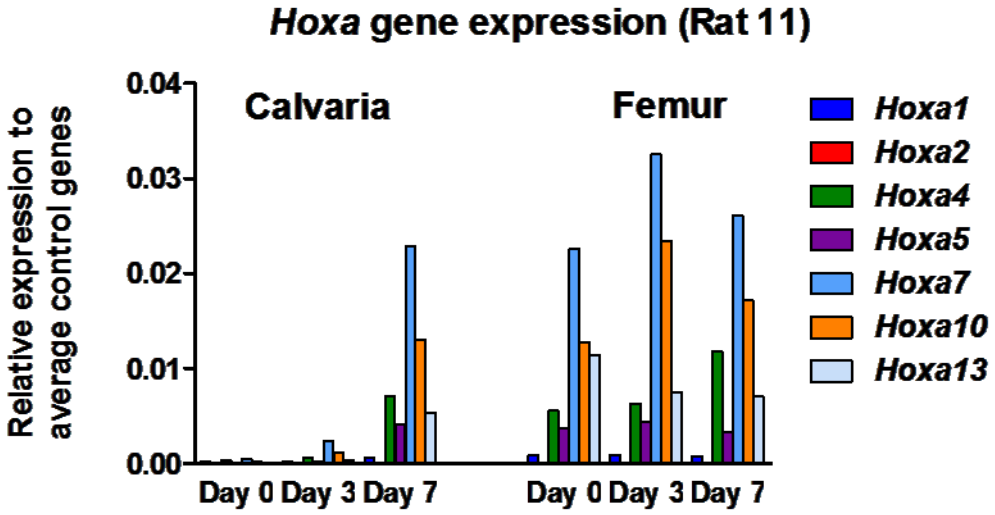


Figure 5-16: Data from Rat 11 in heterotypic culture. The calvarial osteoblastic cells were stained green, whilst femoral osteoblastic cells were unstained. *Vice versa*, calvarial osteoblastic cells were unstained, whilst femoral osteoblastic cells were stained green. Then cells stained cells were sorted for RNA extraction, cDNA synthesis and qRT-PCR. *Hoxa* gene expression was switched on in calvarial osteoblastic cells. At Day 7 in heterotypic culture, *Hoxa* gene expression pattern was observed. *Hoxa7* expressed highest, followed by *Hoxa10* and *Hoxa13*. Data were generated from using 2 primary different cell cultures and 4 replicates were tested for each culture.

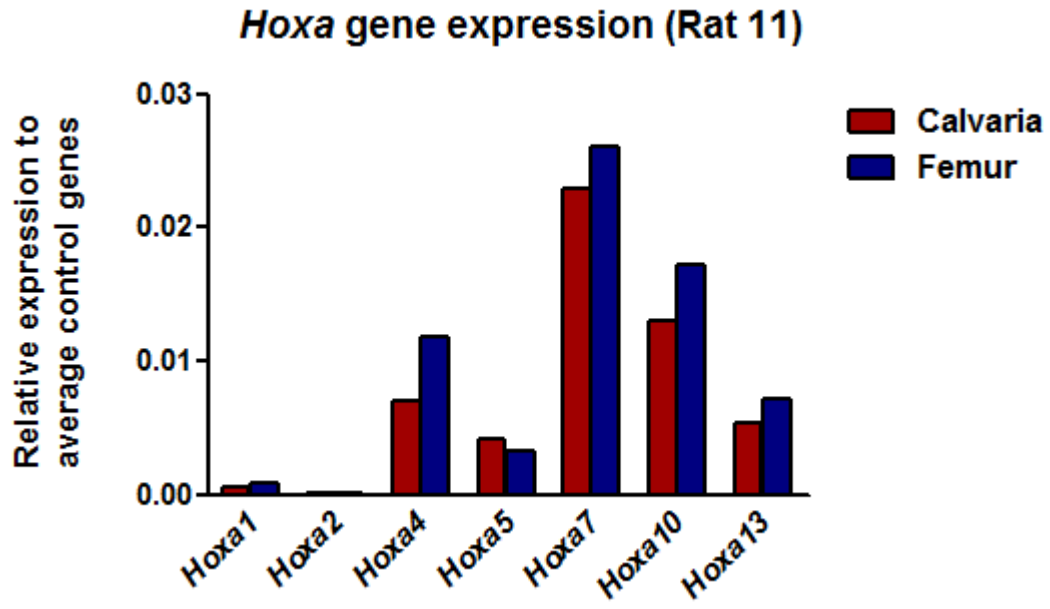


Figure 5-17: Data from Rat 11 calvarial osteoblastic cells were stained green in heterotypic culture when looked closely at Day 7, *Hoxa* gene expression was switched on in calvarial osteoblastic cells. *Hoxa* gene expression pattern was observed. *Hoxa7* expressed highest, followed by *Hoxa10*, *Hoxa4* and *Hoxa13*. Data were generated from using 2 primary different cell cultures and 4 replicates were tested for each culture.

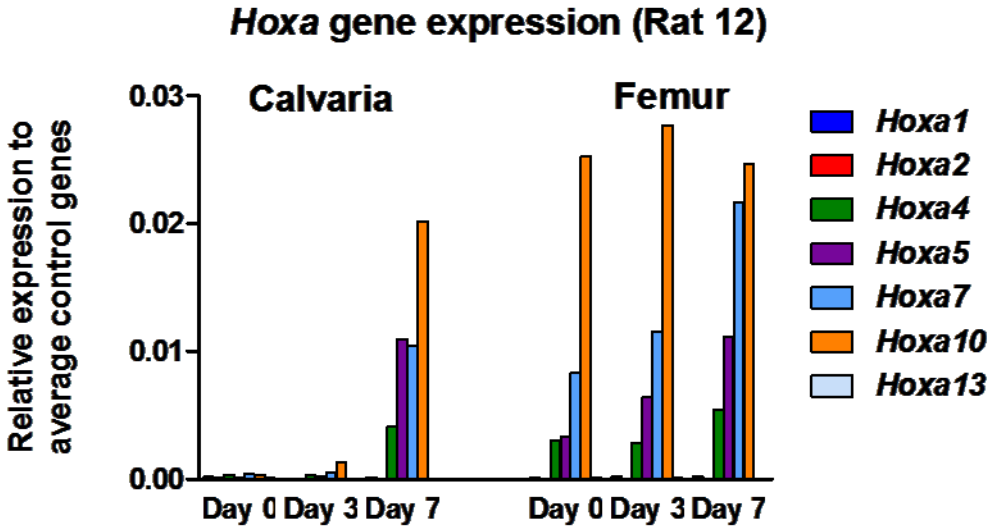


Figure 5-18: Similar results were also observed in Rat 12 in heterotypic culture. The calvarial osteoblastic cells were stained green, whilst femoral osteoblastic cells were unstained. *Vice versa*, calvarial osteoblastic cells were unstained, whilst femoral osteoblastic cells were stained green. Then cells stained cells were sorted for RNA extraction, cDNA synthesis and qRT-PCR. *Hoxa* gene expression was switched on in calvarial osteoblastic cells. At Day 7 in heterotypic culture, *Hoxa* gene expression pattern was observed. Instead, *Hoxa10* expressed highest, followed by *Hoxa7*, and *Hoxa5*, but no *Hoxa13* gene expression. Data were generated from using 2 primary different cell cultures and 4 replicates were tested for each cell culture.

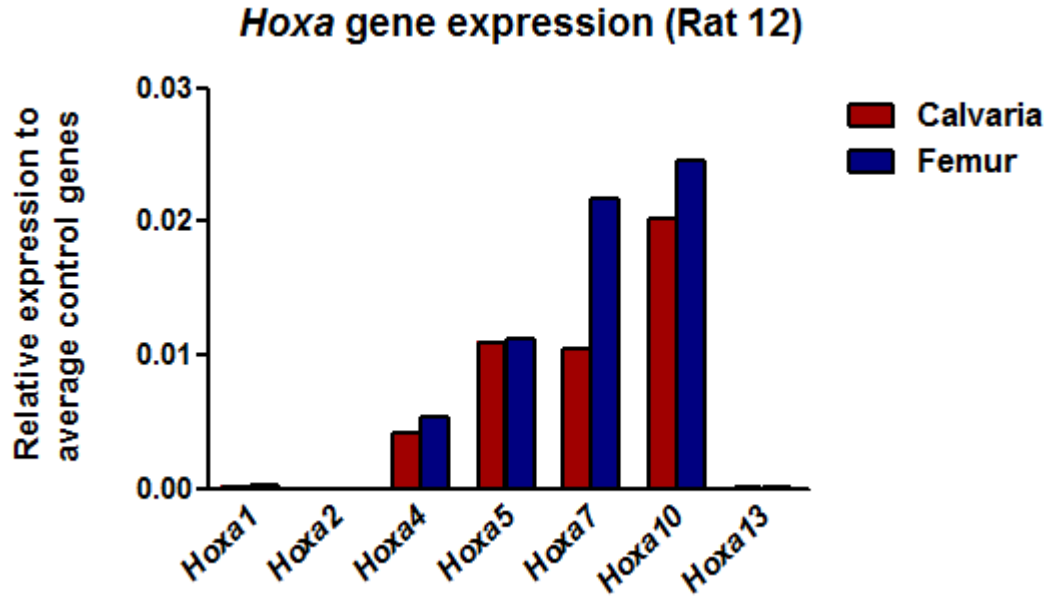


Figure 5-19: Data from Rat 12 calvarial osteoblastic cells were stained green in heterotypic culture when looked closely at Day 7, *Hoxa* gene expression was switched on in calvarial osteoblastic cells. *Hoxa* gene expression pattern was observed. *Hoxa10* expressed highest, followed by *Hoxa5*, *Hoxa7* and *Hoxa4*. Data were generated from using 2 primary different cell cultures and 4 replicates were tested for each culture.

In order to verify this important result, we had to be sure that it is actually changed rather than due to cross contamination of labelled cells. It was crucial to demonstrate that label does not leach from one cell type to the other. Therefore, we carried out further experiments to test for this possibility:

1. Investigation of transfer of label from one cell type to the other

a) FACS analysis of double stained heterotypic cultures

Results showed that the cells labelled green have gradually decreased their fluorescence intensities. Crucially it was observed that by Day 3 many of the previously green labelled cells had become double labelled and picked up red staining in addition to the green label. In contrast, the cells stained with red dye have maintained their fluorescence intensities from Day 0 through Day 3 in culture as shown in flow cytometry data. Double labelling was observed at Day 3 as shown in Figure 5-20.

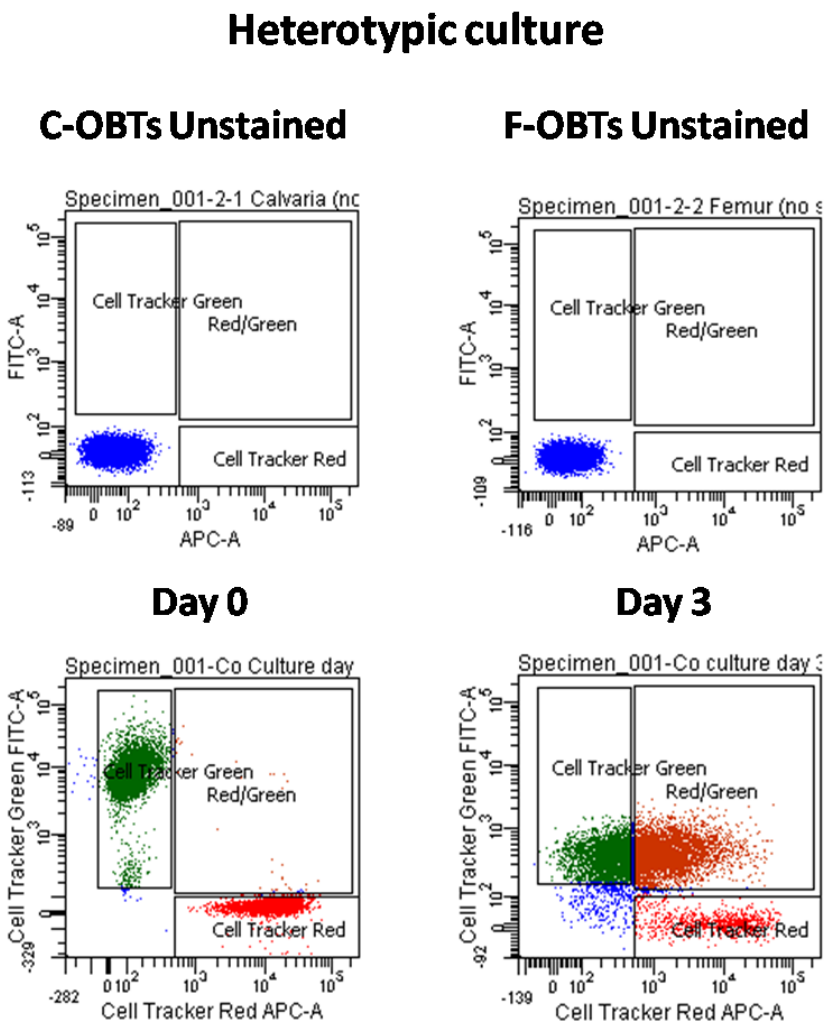


Figure 5-20: Flow cytometry data show two distinct osteoblast populations previously labelled with CellTracker™ Green and CellTracker™ Red and grown in heterotypic culture. They were detected due to their fluorescence intensity signals through FITC-A and APC-A channels. At Day 0, the fluorescence intensity is high, ranging from 10^3-10^5 in both colours. APC-A was maintained in both days. In contrast, at Day 3 FITC-A decreased from 10^4-10^5 to 10^2-10^3. Double labelling was also observed at Day 3 (C-OBTs= Calvarial osteoblasts, F-OBTs= Femoral osteoblasts).

b) BD Falcon™ Cell culture inserts system and flow cytometry

The Falcon insert cultures system to investigate soluble passing of label from separated cell populations was used to check whether one cell population became labelled by another. The results showed that red and orange dyes tended to lose their labels with time in culture. There was some evidence that a small number of orange labelled cells appeared to also acquire green labelling by day 7 (Figure 5-21).

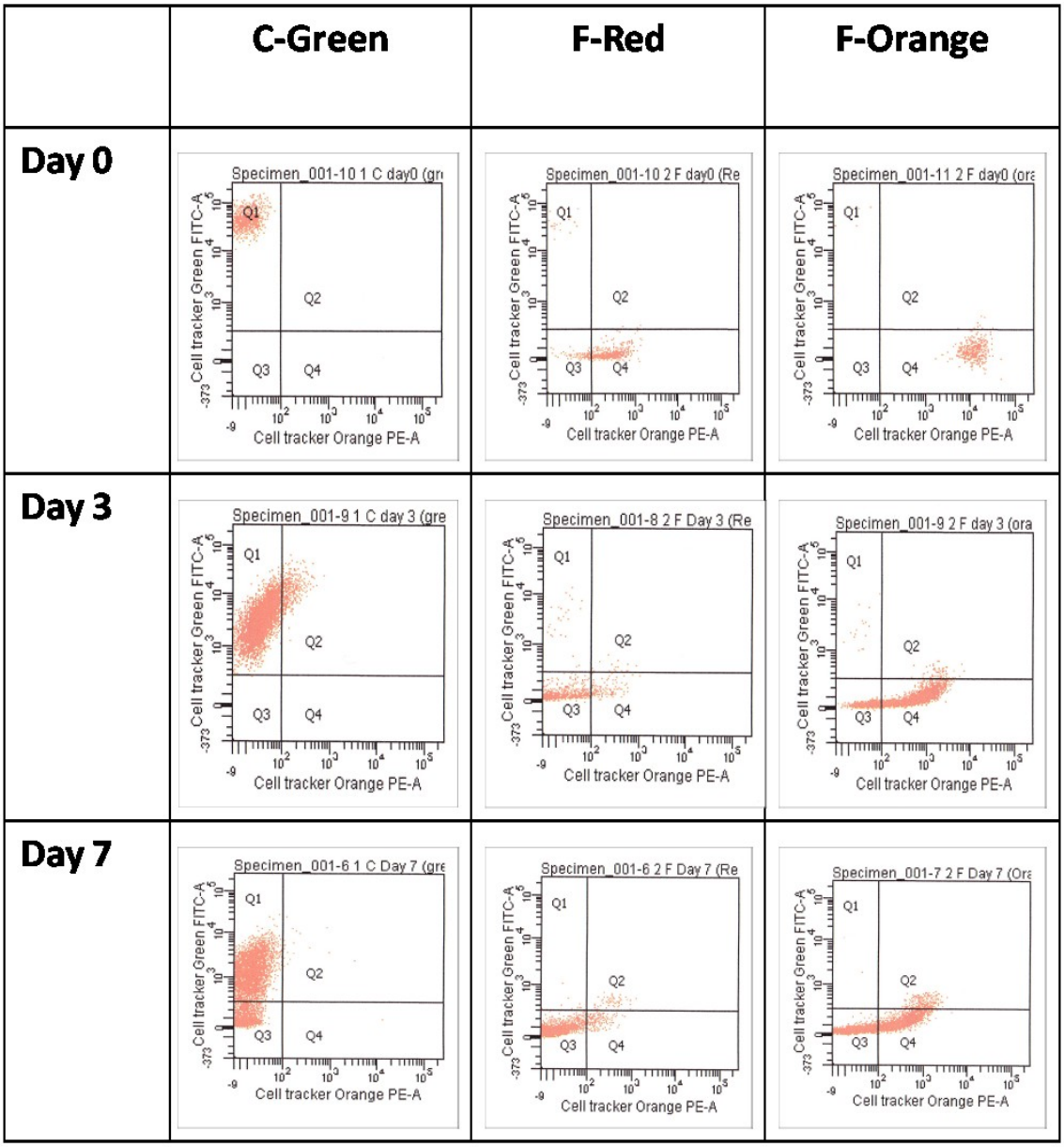


Figure 5-21: The calvarial osteoblastic cells stained with CellTracker™ Green, whilst the femoral osteoblastic cells either stained with CellTracker™ Red or CellTracker™ Orange. They were grown together in BD Falcon™ Cell culture inserts system. The two different cell populations were cultured for 0, 3 and 7 days. At each time point, the cells were collected and tested for dye leakage by using flow cytometry for analysis. The CellTracker™ Green retained the dye in stained cells throughout 7 days culture period. The CellTracker™ Orange retained the dye in stained cells at Day 0, but not at Day 3 and Day 7. Interestingly, the CellTracker™ Red did not retain in stained cells. Double positive labelled was observed in red and orange dyes at Day 3 and Day 7 (C= Calvarial osteoblasts, F= Femoral osteoblasts).

c) BD Falcon™ Cell culture inserts system and qRT-PCR

In addition to dye leakage testing, in this set up we collected the cells which were grown in this system and assessed them for *Hoxa* gene expression. The results showed that *Hoxa* gene expression did not change in this co-culture system, without cell to cell contact (Figures 5-22 and 5-23).

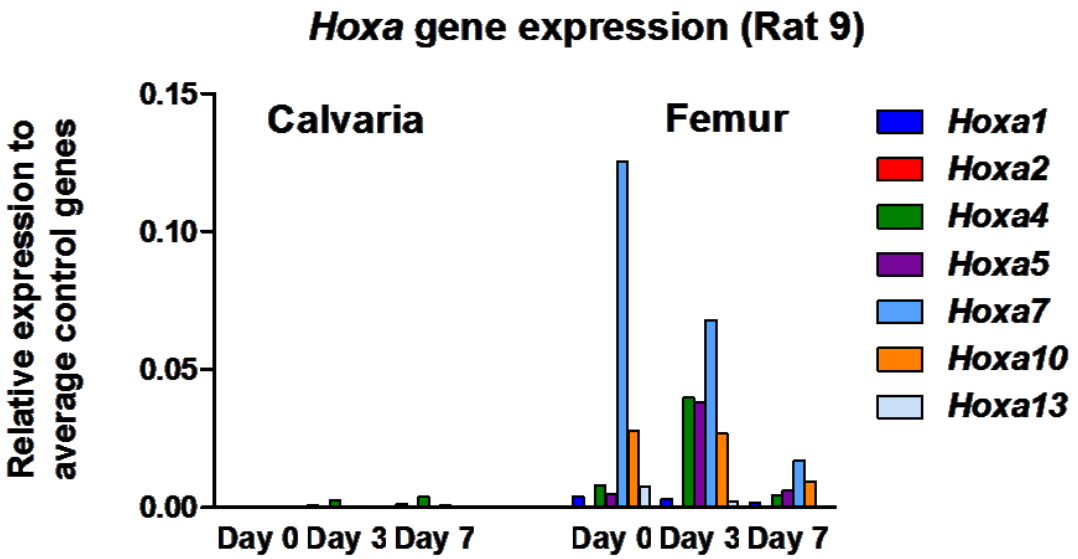


Figure 5-22: The calvarial and femoral osteoblastic cells from Rat 9 were stained with green and red dyes, respectively. They were grown in this system. *Hoxa* gene expression was assessed as previously described. Hox-ve cells retain negative, whilst Hox+ve cells retain positive and showed pattern of *Hoxa* gene expression.

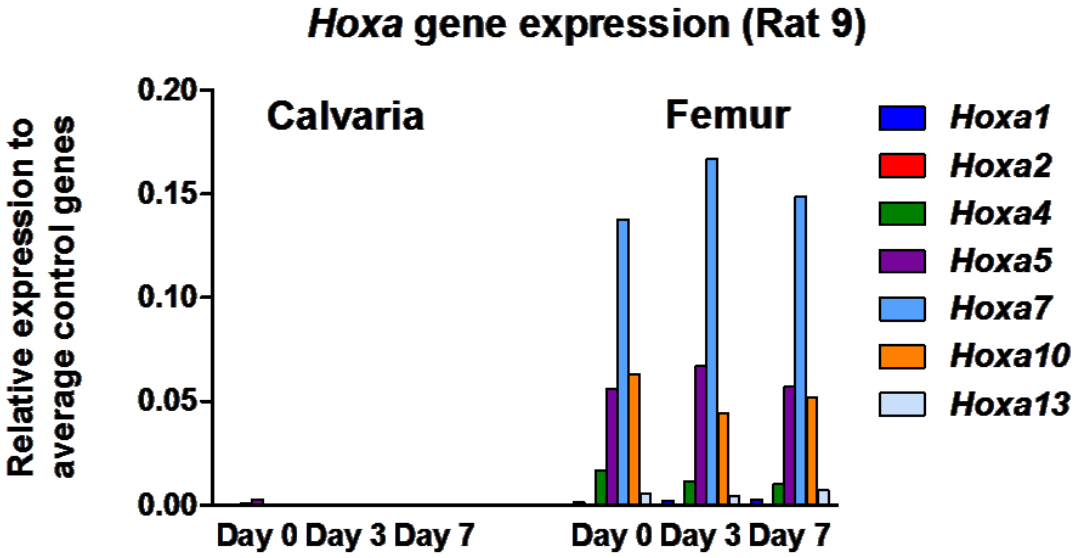


Figure 5-23: The calvarial and femoral osteoblastic cells from Rat 9 were stained with green and orange dyes, respectively. They were grown in this system. *Hoxa* gene expression was assessed as previously described. Hox-ve cells retain negative, whilst Hox+ve cells retain positive and showed pattern of *Hoxa* gene expression.

d) Using CellTracker™ Green only and flow cytometry

As green labelling appeared to be the most stable of the dyes, and showed little evidence of leakage in heterotypic cultures analysed by flow cytometry, it was decided to continue further experiments using green labelling only. The cells were stained, grown together and tested for dye leakage by flow cytometry. The green labelled cells retained their labels in monotypic culture (C or F) and heterotypic culture (C/F) up to 7 days. The two populations of stained and unstained cells can be clearly distinguished (Figure 5-24).

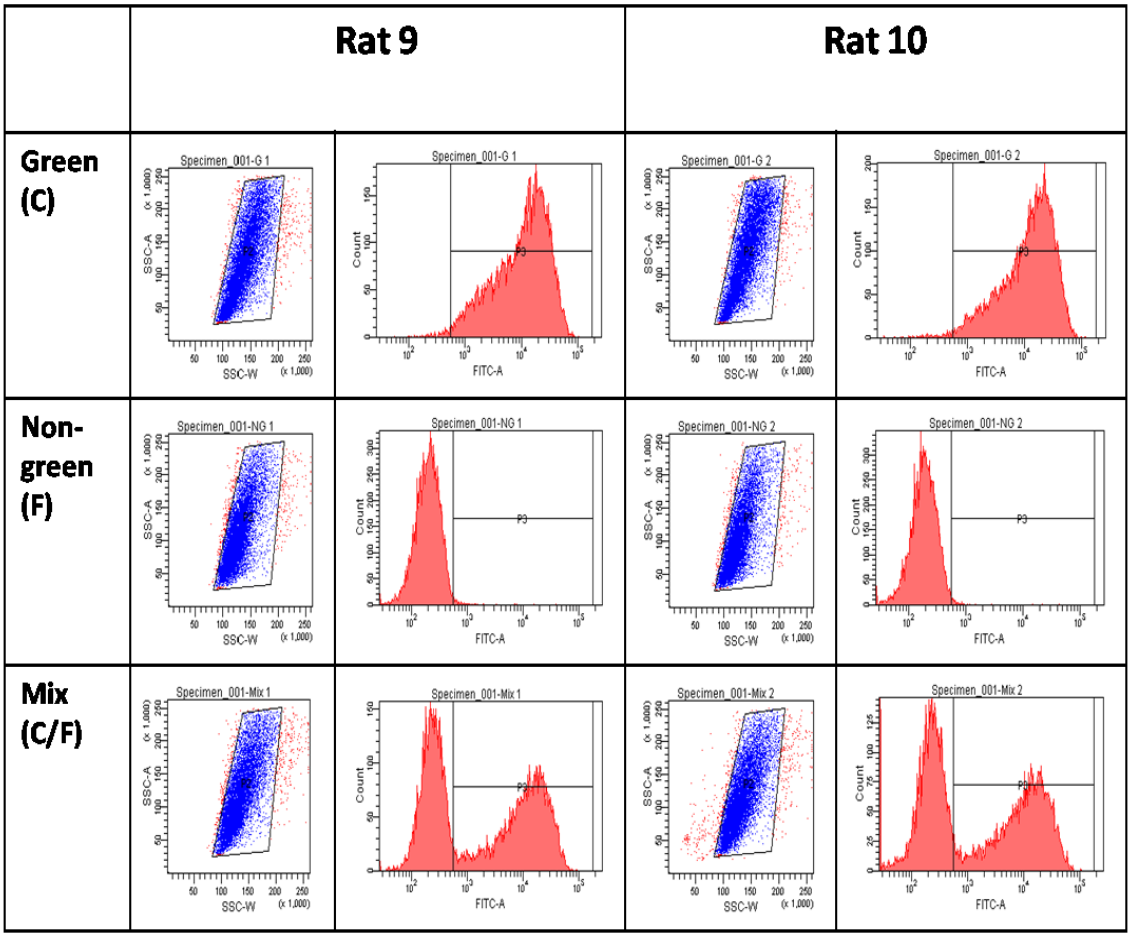


Figure 5-24: The calvarial and femoral osteoblastic cells from 2 rats (Rat 9 and Rat 10) were grown, stained and tested for dye leakage by flow cytometry. The CellTracker™ Green retained the staining in the cells both in monotypic culture (C or F) and heterotypic culture (C/F) up to 7 days. The two populations of stained and unstained cells can be clearly distinguished.

e) Flow cytometry sorting- heterotypic culture

The cells were grown, stained and sorted as previously described in section 5.3.3. The rat calvarial osteoblastic cells stained with CellTracker™ Green from 4 rats have gradually decreased their fluorescence intensities. However, the cell sorter can clearly distinguish stained-cells *versus* unstained from Day 0 through Day 7 in heterotypic culture as shown in Figure 5-25.

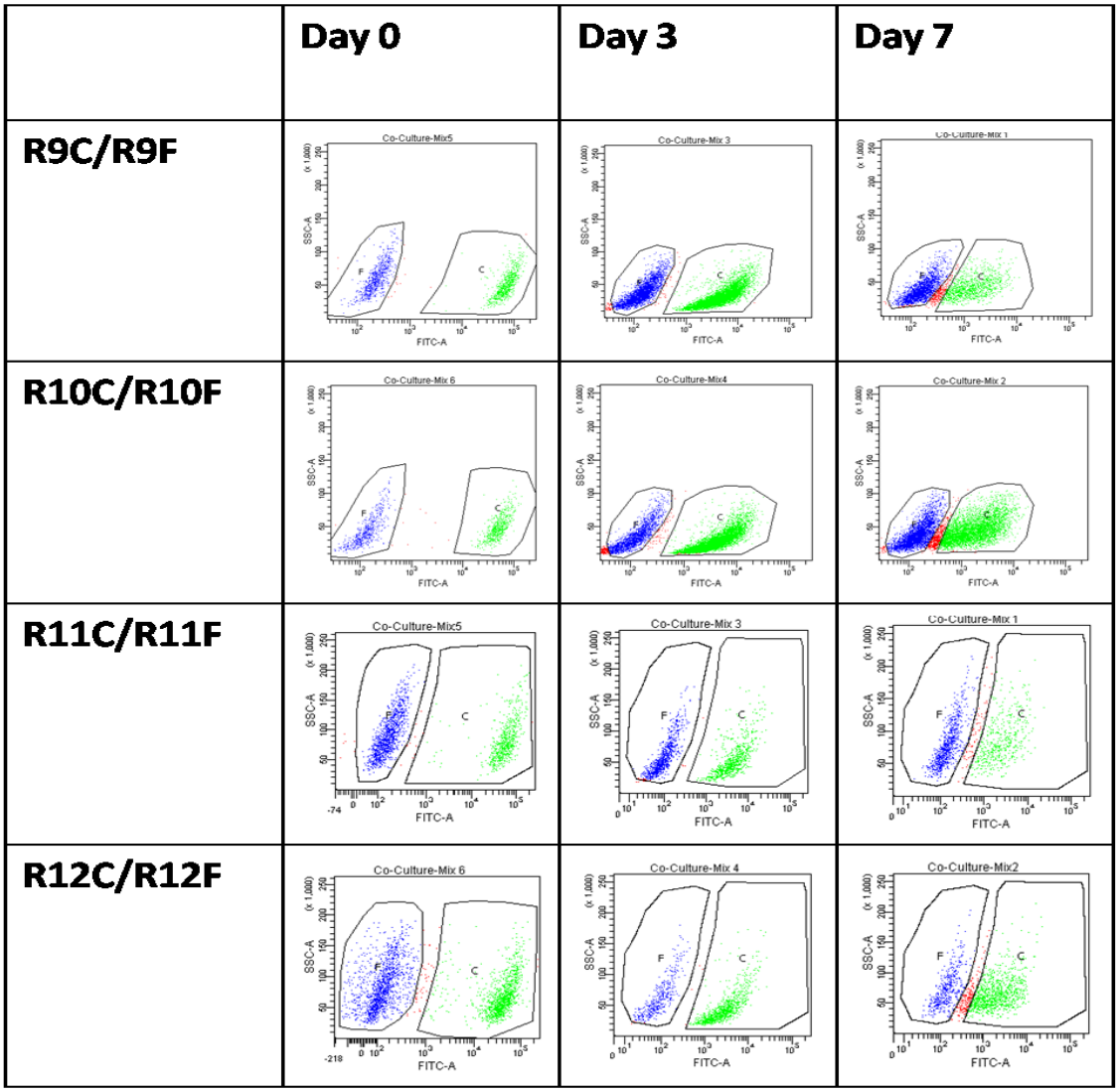


Figure 5-25: Rat calvarial and femoral osteoblastic cells from 4 male rats (Rats 9-12) were grown and used at passage 6-9 in experiments. Calvarial osteoblastic cells were stained with CellTracker™ Green, whilst femoral osteoblastic cells were unstained. The two different populations were grown together in heterotypic culture for 0, 3 and 7 days. Then they were sorted by cell sorter (BD FACSAria II) to separate two different cell populations. The rat calvarial osteoblastic cells stained with CellTracker™ Green from 4 rats have gradually decreased their fluorescence intensities (FITC-A from 10^5 to 10^3). However, the cell sorter can clearly distinguish stained-cells *versus* unstained femoral osteoblastic cells that maintained their low fluorescence intensities (FITC-A 10^2) from Day 0 through Day 7 in heterotypic culture.

Overall the results suggest that when two colours were used in heterotypic culture, the cells labelled green become red, especially at Day 7. It appears that green dye is stable, but red dye is not.

Therefore, we carried out the rest of the experiments using one colour label. We considered that the cells labelled with green dye become unlabeled with time in culture. However, we were confident that sorted green labelled populations were neither contaminated nor a mixed population as the purity checked after sorting was more than 96%. Nevertheless, the unlabelled cells would be a mixture of populations particularly with time in culture, as some green labelled cells would lose their staining. Therefore, we developed the heterotypic culture model with a single stain, green only.

5.4.5 Experiments using single labelling to show that heterotypic cultures do not induce changes in *Hoxa* gene expression

5.4.5.1 Calvarial osteoblastic cells stained with CellTracker™ Green

When we set up monotypic culture and used as controls, as expected gene expression did not change in monotypic culture (Figure 5-26).

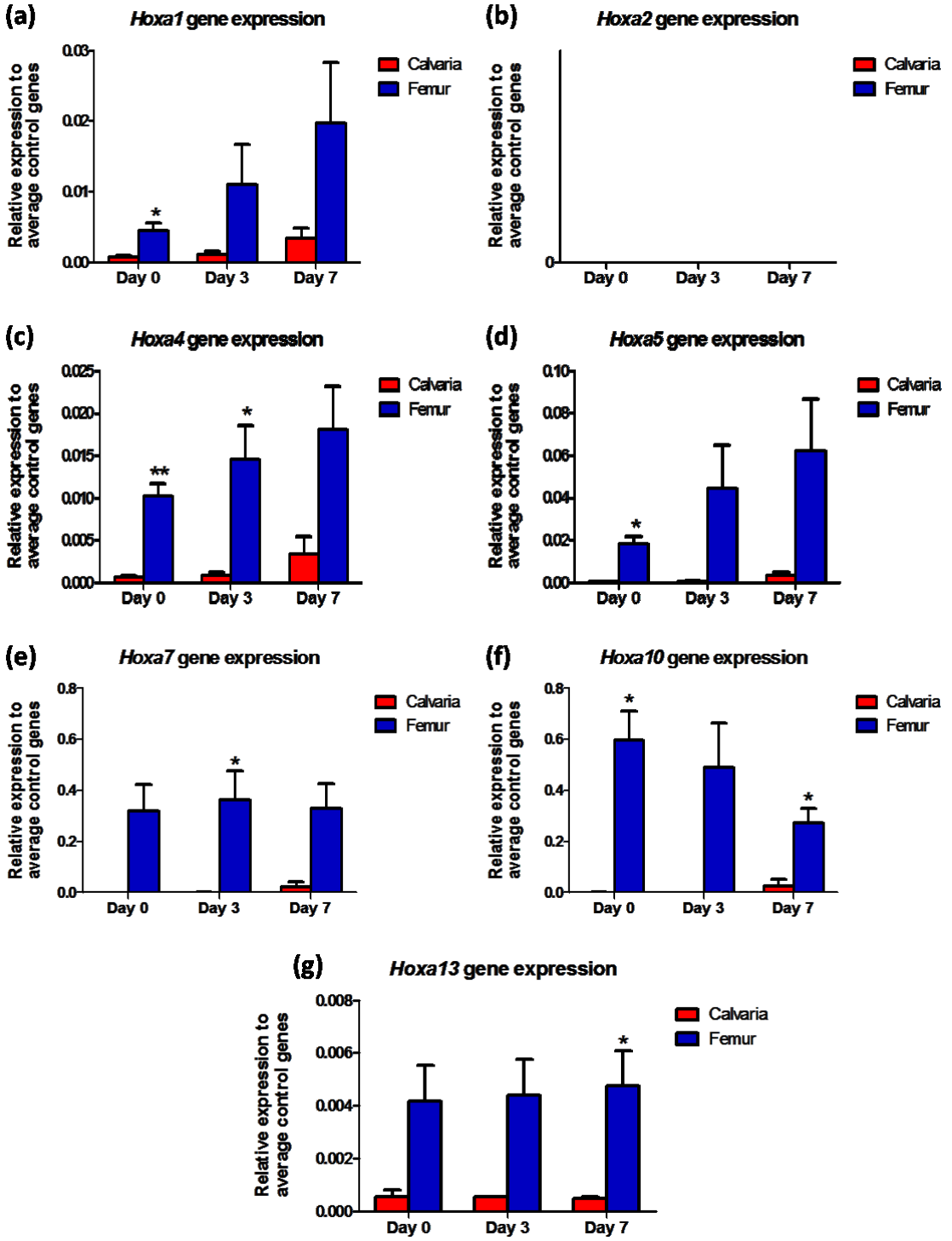


Figure 5-26: Relative expression of *Hoxa* genes to average control genes (*Atp5b* and *Eif4a2*). In monotypic culture, calvarial osteoblastic cells stained with CellTracker™ Green, whilst femoral osteoblastic cells were unstained. At Day 0, Day 3 and Day7, the cells passed through cell sorter, grown and collected for RNA extraction, cDNA synthesis and qRT-PCR. Data represented as mean ± SD from 4 different male rats matched pairs. Data were generated from using 8 different primary osteoblast cultures and 4 replicates were tested for each culture. No or nearly undetectable level of *Hoxa* gene expression in calvarial osteoblastic cells (a-g). In contrast, there is a pattern of *Hoxa* gene expression in femoral osteoblastic cells (a-g), especially *Hoxa7* (e) and *Hoxa10* (f). Paired *t*-test was used to compare gene expression between calvarial and femoral osteoblastic cells, *, $p<0.05$, **, $p<0.01$ considered significantly different.

In heterotypic cultures using single labelling the results obtained are as followed:

The experiment was repeated two times independently. The same results were obtained. There was no or nearly undetectable levels of *Hoxa* gene expression in calvarial osteoblastic cells (Figure 5-27). In contrast, there is a pattern of *Hoxa* gene expression in femoral osteoblastic cells consistent with our results from Chapter 4. *Hoxa7* (Figure 5-27 e) and *Hoxa10* (Figure 5-27 f) were highly expressed. A detectable level of *Hoxa4* and *Hoxa5* gene expression was observed. In addition, low levels of *Hoxa1* and *Hoxa13* gene expression was seen and no expression of *Hoxa2* gene. Interestingly, at Day 0 *Hoxa5* and *Hoxa7* were expressed at significantly higher levels, but gradually decreased with time in culture (Figures 5-27 d and e).

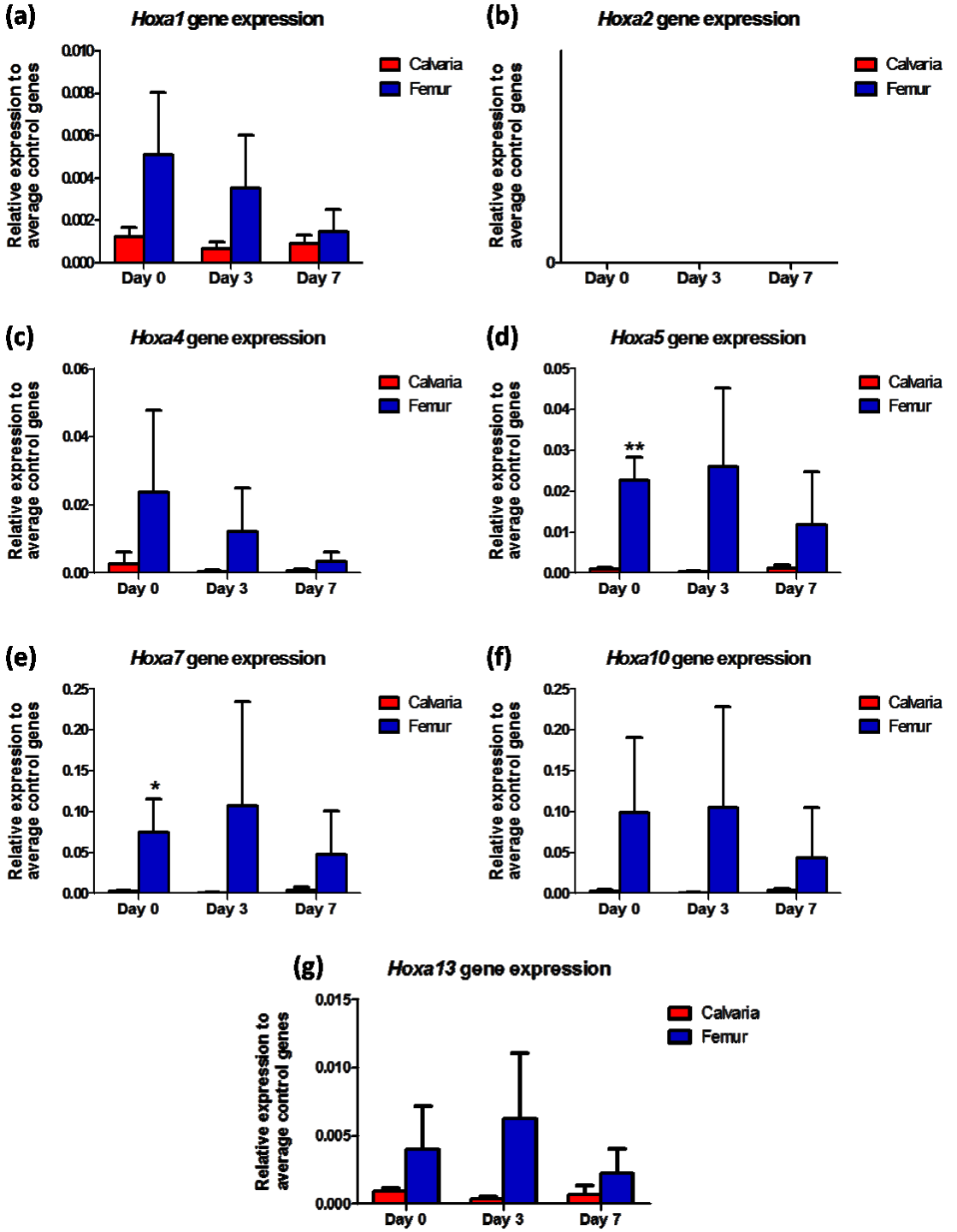


Figure 5-27: Relative expression of *Hoxa* genes to average control genes (*Atp5b* and *Eif4a2*). In heterotypic culture, calvarial osteoblastic cells stained with CellTracker™ Green, whilst femoral osteoblastic cells were unstained. The cells were grown together in culture for 0, 3 and 7 days. Then they were sorted by cell sorter, continued to grow and collected for RNA extraction, cDNA synthesis and qRT-PCR. Data represented as mean ± SD from 4 different male rats. Data were generated from using 8 different primary osteoblast cultures and 4 replicates were tested for each culture. No or nearly undetectable level of *Hoxa* gene expression in calvarial osteoblastic cells (a-g). In contrast, there is a pattern of *Hoxa* gene expression in femoral osteoblastic cells (a-g), especially highly expressed *Hoxa7* (e) and *Hoxa10* (f). Paired *t*-test was used to compare gene expression between calvarial and femoral osteoblastic cells. *,$p<0.05$, **,$p<0.01$ considered significantly different.

5.4.5.2 Femoral osteoblastic cells stained with CellTracker™ Green

When we set up monotypic culture and used as controls, as expected gene expression did not change in monotypic culture (Figure 5-28).

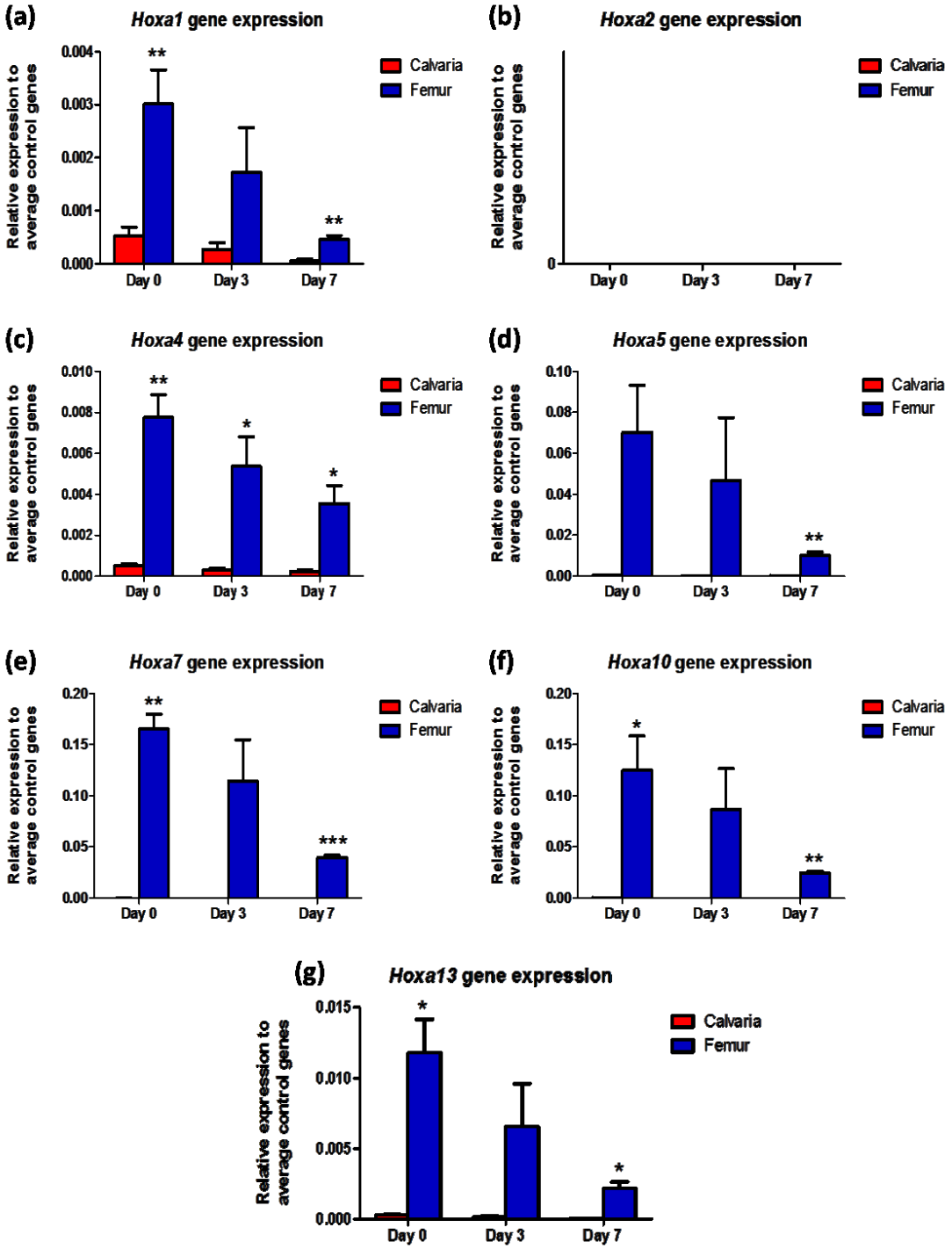


Figure 5-28: Relative expression of *Hoxa* genes to average control genes (*Atp5b* and *Eif4a2*). In monotypic culture, femoral osteoblastic cells stained with CellTracker™ Green, whilst calvarial osteoblastic cells were unstained. At Day 0, Day 3 and Day7, the cells passed through cell sorter, grown and collected for RNA extraction, cDNA synthesis and qRT-PCR. Data represented as mean ± SD from 4 different male rats matched pairs. Data were generated from using 8 different primary osteoblast cultures and 4 replicates were tested for each culture. No or nearly undetectable level of *Hoxa* gene expression in calvarial osteoblastic cells (a-g). In contrast, there is a pattern of *Hoxa* gene expression in femoral osteoblastic cells (a-g), especially *Hoxa7* (e) and *Hoxa10* (f). Paired *t*-test was used to compare gene expression between calvarial and femoral osteoblastic cells, *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$ considered significantly different.

Femoral osteoblastic cells were stained green. Similar results with single staining were obtained when calvarial osteoblastic cells were stained green. There was no or nearly undetectable level of *Hoxa* gene expression in calvarial osteoblastic cells (Figure 5-29).

In contrast, there is a pattern of *Hoxa* gene expression in femoral osteoblastic cells, especially high expression of *Hoxa7* (Figure 5-29 e) and *Hoxa10* (Figure 5-29 f). Detectable levels of *Hoxa4* and *Hoxa5* gene expression were observed. In addition, low levels of *Hoxa1* and *Hoxa13* gene expression was seen and no expression of *Hoxa2*.

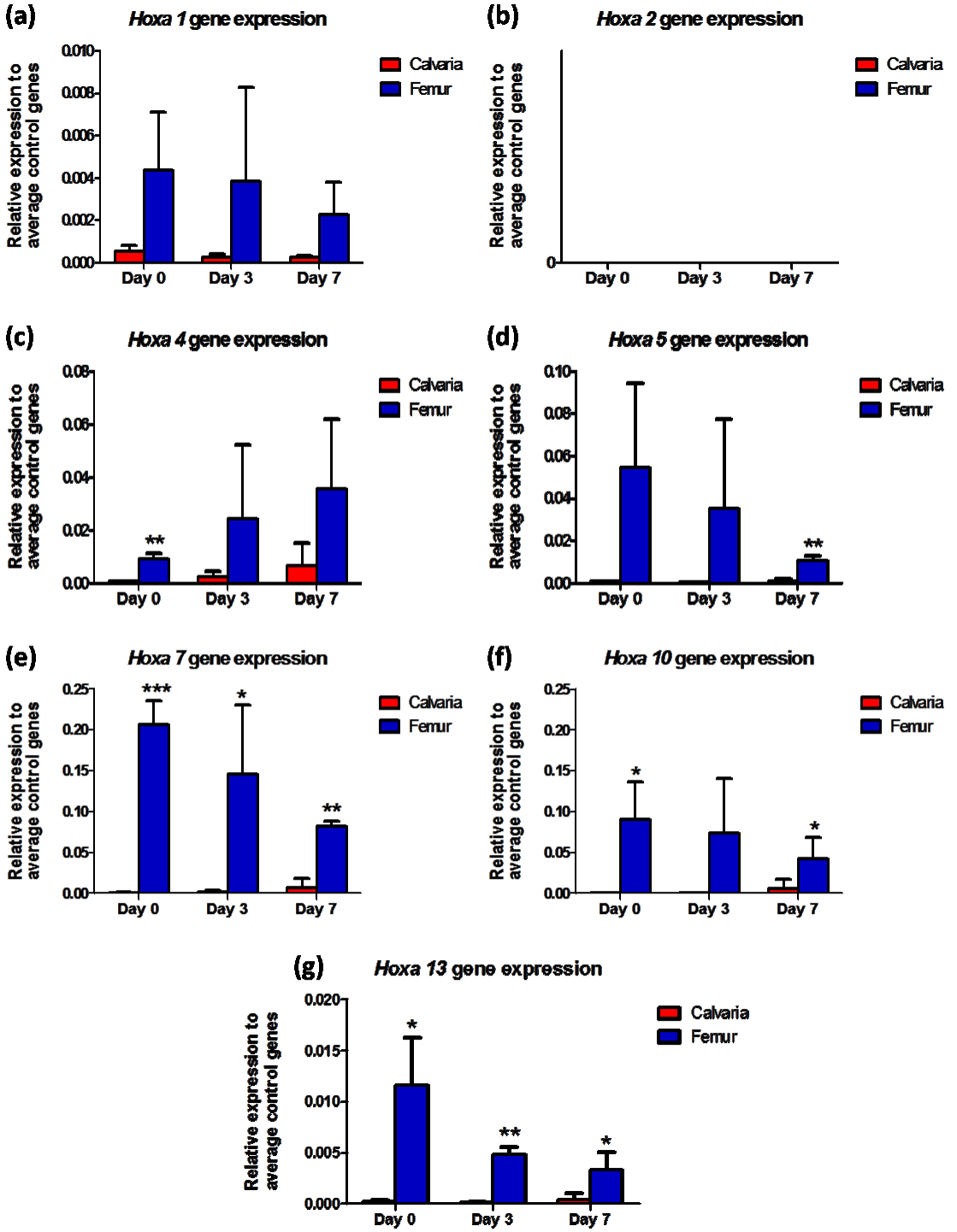


Figure 5-29: Relative expression of *Hoxa* genes to average control genes (*Atp5b* and *Eif4a2*). In heterotypic culture, femoral osteoblastic cells stained with CellTracker™ Green, whilst calvarial osteoblastic cells were unstained. The cells were grown together in culture for 0, 3 and 7 days. Then they were sorted by cell sorter, continued to grow and collected for RNA extraction, cDNA synthesis and qRT-PCR. Data represented as mean ± SD from 4 different male rats. Data were generated from using 8 different primary osteoblast cultures and 4 replicates were tested for each culture. No or nearly undetectable level of *Hoxa* gene expression in calvarial osteoblastic cells (a-g). In contrast, there is a pattern of *Hoxa* gene expression in femoral osteoblastic cells (a-g), especially highly expressed *Hoxa7* (e) and *Hoxa10* (f). Paired *t*-test was used to compare gene expression between calvarial and femoral osteoblastic cells. *,$p<0.05$, **,$p<0.01$, ***,$p<0.001$ considered significantly different.

5.5 Discussion and conclusion

From the previous study by Leucht and co-workers using *Wnt1Cre::Z/EG* transgenic mice, testing for embryonic origin of the graft influences cell fate decision in a transplantation assay using Pentachrome staining and GFP antibody staining, they have found that homotopic transplantation of *Hoxa11*-expressing periosteum cells derived from tibia into tibial defects resulted in vigorous bone formation through intramembranous ossification at post-surgical day 10, and homotopic graft of mandibular periosteum into a mandibular injury site induced direct differentiation into bone (Leucht et al., 2008a).

In addition, the majority of the regenerated tissue was derived from the GFP-positive grafted periosteum. Interestingly, placing neural crest-derived periosteum into a mesoderm-derived injury site resulted in intramembranous bone formation, but placing tibial periosteum into a mandibular injury site resulted in endochondral ossification and underwent hypertrophy even though the environment fully supported osteogenic differentiation (Leucht et al., 2008a). It was concluded that the disparity between the Hox status of the graft and the injury site correlated with a disruption in bone regeneration as the grafted cells failed to differentiate into osteoblasts.

The results of that mouse study *in vivo* suggested that Hox-positive host cells might be responsible for this switch in molecular fate. In contrast, we thought that the switch could be due to other growth factors or soluble factors in their new environment or the committed cell itself.

To address the first question, we first established our rat dermal fibroblasts from scalp which previously tested Hox-negative, and rat dermal fibroblasts from tissue overlying ulna area which previously tested Hox-positive. We carried out the experiment to test the effect of soluble factors on *Hoxa* gene expression in rat dermal fibroblasts by treating Hox-negative and Hox-positive fibroblasts with different condition media.

However, we have found that there was no effect of soluble factors on *Hoxa* gene expression in our rat dermal fibroblasts model when Hox+ve fibroblasts were treated

with different CM. A fold change of 1 indicates no difference from control as shown in Figure 5-6. Therefore, the soluble factors are not responsible for the Hox-switching. The Hox-switching may be due to cell to cell contact. To address the second question, we carried out these monotypic and heterotypic experiments by staining two distinct embryonic origin osteoblasts with CellTracker^{TM} dye, sorted and assessed their *Hoxa* gene expression and also *Hoxa* gene expression pattern by qRT-PCR.

We have found very interesting data from our pilot experiment from Rat 9, Rat 11 and Rat 12 that *Hoxa* gene expression in calvarial osteoblastic cells could be switched on by heterotypic culture. However, femoral osteoblastic cells in Rat 9 after being grown in heterotypic culture for 7 days demonstrated low level of *Hoxa* gene expression and became like calvarial osteoblastic cells (Figure 5-14 and 5-15), but not in femoral osteoblastic cells of Rat 11 (Figure 5-16 and 5-17) and Rat 12 (Figure 5-18 and 5-19) which still maintained their *Hoxa* genes expression in these heterotypic cultures. To be certain of this result, we considered other possibilities that might cause this modulation. Is it due to dye leakage in heterotypic culture? Or the cells were dead and the dye was released, then some live cells that lost their labelling picked up the dye which caused cell contamination and false modulation.

We tested our system by growing our cells in BD Falcon™ Cell culture inserts system, checking dye leakage by labelling and looking under fluorescence microscope, analysing by using flow cytometry, optimising the staining condition and checking for purity of cells after sorting and the effect of cell sorter on our rat osteoblastic cells.

We have demonstrated that there was no effect of cell sorting on our rat calvarial and femoral osteoblastic cells after they were grown in heterotypic culture for 7 days and then sorted by flow cytometry. The cells from all cultures showed normal morphology under microscope. Many previous studies have also used fluorescence activated cell sorting (FACS) for flow cytometric analysis and cell sorting purposes (Fu et al., 1999; Herbertson and Aubin, 1997; Kulterer et al., 2007; Lopes et al., 1998; Manz et al., 1995; Nakano et al., 1989; Purpura et al., 2003; Zohar et al., 1997). Rat BMSCs could be sorted due to their high or low alkaline phosphatase (AP) expression (Herbertson and Aubin, 1997). Calvarial cells from 21-day-old foetuses of timed-pregnant Wistar rats and rat or mouse BMSCs were studied for their cell vitality, OPN mRNA expression,

alkaline phosphatase activity and cell proliferation, limiting dilution analysis, colony assays, self-renewal capacity, electron microscopy and immunostaining after the cells passed through flow cytometer and sorting (Zohar et al., 1997). It was concluded that the method of flow cytometry sorting provided a simple, reproducible approach to help define the stages of bone cell differentiation and ability to enrich for stem cells.

When our rat osteoblastic cells were stained with different colours of CellTracker™ dye, we have found that CellTracker™ Red caused a dye leakage problem analysed by flow cytometry showing doubling labelling. We have also found the CellTracker™ Orange has decreased fluorescence intensity overtime during heterotypic culture in BD Falcon™ cell culture inserts system and also double labelling. However, the CellTracker™ Green was the most stable. There was little sign of dye leakage. Nevertheless, CellTracker™ Green showed decreased fluorescence intensity over time during heterotypic culture.

CellTracker™ Green was our best option to use for further experiments. Taking the limitation of losing fluorescence intensity overtime in culture into consideration, we have designed and carried out these following experiments for both monotypic and heterotypic cultures by using CellTracker™ Green only to stain calvarial osteoblastic cells and femoral osteoblastic cells, one colour was used to stain in monotypic or heterotypic cultures. For example, if calvarial osteoblastic cells were stained with CellTracker™ Green, femoral osteoblastic cells were left unstained and *vice versa*. We repeated our experiments by using 4 male rats. This time, we can solve a dye leakage or a cell contamination problem, but the *Hoxa* gene expression in calvarial osteoblastic cells could not be switched on by heterotypic culture as previously discovered. Having repeated these experiments we propose that our initial results suggestive of switching on of *Hoxa* expression in calvarial cells in co-culture were likely to have been the result of dye leakage and transfer.

These results appear to be in contrast to the study by Leucht et al., who found that posterior Hox genes were expressed in Hox-negative adult neural crest cells when placed into a Hox-positive environment, implying that injury sites have specific Hox codes. In contrast, Hox status was maintained in mesodermal cells when grafted into the mandible and failed to differentiate into osteoblasts *in vivo* (Leucht et al., 2008a). In

addition, within 5 days in *in vitro* co-cultured of tibial periosteal *Hoxa11*-expressing cells with Hox-negative mandibular periosteal cells, the formerly Hox-negative mandibular periosteal cells began to express *Hoxa11 in vitro* (Leucht et al., 2008a).

In conclusion, there was no effect of conditioned medium from heterotypic Hox+ve fibroblasts on each other's gene expression as determined by qRT-PCR at least in this time frame. Similarly when osteoblasts were placed in heterotypic cultures, there was no evidence of *Hoxa* switching. In our pilot study, *Hoxa* genes in calvarial osteoblastic cells could be switched on by heterotypic culture, but latter repeated experiments confirmed no modulation in *Hoxa* gene expression in osteoblastic cells due to heterotypic culture. From our data, there was no modulation of positional identity in heterotypic culture. *Hoxa5*, *Hoxa7* and *Hoxa10* are the set of stable key gene throughout the study. Our data also suggested that *Hoxa* in the more anterior region are more flexible than in the posterior region.

Chapter 6

Chapter 6: Phenotypic differences of regionally distinct osteoblasts

6.1 Introduction

In Chapter 4, it was demonstrated that cells derived from distinct anatomical sites showed differences in *Hoxa* gene expression consistent with their embryonic origins. These differences in positional identity persisted and were retained *in vitro* for at least up to 10 subcultures. These observations raise the important question of whether regionally distinct osteoblasts may also exhibit inherent functional differences as well.

As previously discussed, Rawlinson et al. found the embryological development associated genes (in skull such as *Zic*, *Dlx*, *Irx*, *Twist1* and *Cart1*; in Limb such as *Hox*, *Shox2*, and *Tbx* genes) in both adult bones and isolated adult bone-derived cells showed the differentially expression (Rawlinson et al., 2009b). By using gene expression microarrays they found that skull bone-derived cells preferentially expressed genes associated with the craniofacial development of neural crest derived cells, whilst genes for limb development and patterning were preferentially expressed by limb bone-derived cells. In addition, the *Spp1* gene, which codes for the bone matrix protein osteopontin, was more highly expressed in limb bone-derived cells (Rawlinson et al., 2009b).

It is well known (Albright et al., 1941; Ralston and de Crombrugghe, 2006; Warriner et al., 2011) that limb bones are susceptible to osteoporosis and they become weak and vulnerable to fractures when they are not maintained by weight bearing exercise. However skull bone, which bears almost no weight, remains largely resistant to both post-menopausal osteoporosis and disuse atrophy (Albright et al., 1941; Ralston and de Crombrugghe, 2006; Warriner et al., 2011). However, the molecular and cellular mechanisms mediating this effect remain unclear. These empirical observations support the idea that regionally distinct osteoblasts may exhibit phenotypic differences which may be inherently programmed or alternatively determined by their anatomical location.

Osteopontin (OPN) is an arginine-glycine-aspartate (RGD) - containing glycoprotein encoded by the gene *secreted phosphoprotein1*(*Spp1*) (Liaw et al., 1998). OPN plays an important role in bone formation under tensile mechanical stress (Morinobu et al., 2003). In OPN knockout mice, bone remodelling in response to mechanical stress was suppressed. These results indicate the essential role of OPN in the process of bone

remodelling (Fujihara et al., 2006). In addition, OPN expression is increased in osteoblasts subjected to mechanical stimulation (Kubota et al., 1993).

Osteoblasts derived from ulnae and cultured on plastic plates treated with dynamic strain of 600 cycles (1 Hz, 4000 mu epsilon) showed increasing in G6PD activity. There was not the same response in calvarial-derived cells even in similarly treated. Calvarial-derived bone cells do not respond to physiological strains in their regions with increased prostanoid release or G6PD activity either *in situ* or when seeded onto dynamically strained plastic plates which differ from those of the ulna (Rawlinson et al., 1995).

Compressive forces act on bone cells depend on the magnitude and provide a possible molecular model for the increasing bone strength observed in response to physical activity (Rath et al., 2008). The study in biomechanical forces suggests that these forces are significant to develop fracture healing.

Mice with ablated OPN gene do not lose bone after mechanical unloading (tail suspension) (Ishijima et al., 2001) or upon oestrogen withdrawal (ovariectomy) (Yoshitake et al., 1999), showing the importance of OPN in the regulation of bone remodelling by osteoblasts and osteoclasts. Also, as previously mentioned, OPN $^{-/-}$ mice do not lose bone with hind-limb unloading, which is an established model of disuse osteoporosis (Ishijima et al., 2001).

The oestrogen and androgen have important roles in maintaining the adult skeleton. ER-related receptor-α (ERRα) is expressed in tissues such as the female and male reproductive organs, mature ovocytes in the ovary, and spermatocytes in the testis; in non-reproductive tissues such as mammary glands, heart, and nervous system (Bonnelye et al., 1997).

In knock-out mice, osteoblast-like cells in primary cultures derived from $ER^{-/-}$ mice do not proliferate in response to mechanical strain, unlike those from $ER^{+/+}$ mice. Data from this study also support the hypothesis that reduction in ERα expression or activity after oestrogen withdrawal results in a less osteogenic response to loading. This may be important in the etiology of postmenopausal osteoporosis (Jessop et al., 2004).

Based on their embryonic origins, adult skeletal progenitor cells from at least two populations can be distinguished. Injured skeletal elements usually repair by using cells of their own embryonic origin (Leucht et al., 2008a). The reparative strategies may have to consider the variable in osteoblastic cells from two distinct populations to improve clinical outcome and healing when using cells from the same embryonic origin.

Overall, potential inherent phenotypic differences between regionally distinct osteoblasts may have important clinical implications in the long term for conditions such as osteoporosis, and in tissue engineering applications. In Chapter 3, we noted some apparent phenotypic differences between calvarial and femoral osteoblastic cells, most notably in bone nodule formation capacity, but also in osteopontin production. In contrast, there was no significant difference seen in osteocalcin production in our cells. In Chapter 5, we concluded that there is no effect of conditioned medium from heterotypic Hox+ve fibroblasts on each other's gene expression as determined by qRT-PCR. In a pilot study, *Hoxa* genes in calvarial osteoblastic cells could be switched on when grown in heterotypic culture. We have repeated these experiments, but could not demonstrate the modulation in *Hoxa* gene expression in osteoblastic cells. There was no modulation of positional identity by heterotypic culture. However, there may be a possibility that phenotypic differences might be modulated and changes such as proliferation, total ALP activity, mineralised bone nodules, transcription factors or osteoblast-associated gene expression might be observed. Therefore in this chapter we explored potential phenotypic differences between these cell types further.

6.2 Aims

The aim of the studies described in this chapter was to investigate if there are phenotypic differences between osteoblasts derived from distinct anatomical sites. Specifically objectives were:

1. To investigate functional differences in osteoblasts derived from femurs and calvariae;

2. To investigate differences in expression of osteopontin protein in osteoblasts from femurs and calvariae *in vitro*;

3. To test for the persistence of osteoblast-associated genes between osteoblastic cells from femurs and calvariae *in vitro*;

4. To test for differences in oestrogen responsiveness between osteoblastic cells from femurs and calvariae *in vitro*;

5. To investigate whether cell to cell contact in heterotypic culture can modify proliferation and total ALP activity, change ability to produce mineralised bone nodule, and modulate expression of transcription factors and osteoblast-associated genes.

6.3 Materials and Methods

6.3.1 Overview

To investigate potential phenotypic differences, matched pairs of femoral and calvarial-derived osteoblastic cultures from 4 male adult rats were used as described previously. Cultures were tested for differences in proliferation rates, alkaline phosphatase activity, production of osteopontin , expression of osteoblast- associated genes; *Runx2*, *Bglap* and *Spp1*, differences in responsiveness to oestrogen stimulation, and finally using single labelling to test whether heterotypic cultures can induce changes in proliferation, total ALP activity, mineralised bone nodules, expression of transcription factors and osteoblast-associated genes.

6.3.2 Proliferation and ALP activity

Calvarial and femoral osteoblastic cells were grown in DMEM with 10% FCS supplemented with 10 ng/ml of FGF-2. At around 80% confluence the cells were tested for proliferation by MTS assay followed by total ALP activity assay. Briefly, 2000 cells of each cell type were seeded in 96-well plates in triplicate and were grown for periods of 7 and 14 days prior to analysis for MTS and total ALP activity.

6.3.3 Expression of osteopontin by ELISA

Production of osteopontin by cultures was assessed by ELISA as described in section 2.7 and 3.4.3.3.

6.3.4 Expression of osteoblast-associated genes

The calvarial and femoral osteoblastic cells were grown in osteoblast culture medium supplemented with 10 ng/ml of FGF-2 to 80% confluence. They were then lysed and collected for total RNA extraction as described in section 2.12. These experiments were carried out with cells at both passage 5 and passage 10. Cells were then tested for expression of *Runx2*, *Bglap* and *Spp1* genes by qRT-PCR as described in section 2.13-2.18.

6.3.5 Oestrogen stimulation

The rat calvarial and femoral cells at passage 3 were taken out from liquid nitrogen, grown in T-75 flasks in DMEM with 20% FCS supplemented with 10 ng/ml of FGF-2 up to 3-5 days. At 80-90% confluence, cells were passaged and grown in T-75 flasks in DMEM with 10% FCS supplemented with 10 ng/ml of FGF-2 up to 5-7 days. At 80-90% confluence, cells were passaged and grown in T-75 flasks in Phenol Red-free DMEM with 10% FCS without FGF-2 for 1 week as described in Figure 6-1.

To measure the effects of oestrogen stimulation on proliferation, a pilot study using cells from 4 rats was carried out. Briefly, after the cells were grown in 10% FCS in Phenol Red-free DMEM for 1 week, the cells were seeded into 96 well plates, serum starved overnight, and stimulated with different concentrations of oestrogen (0, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} M) and dexamethasone 10^{-8} M and grown for 3 and 7 days. Then their responses were measured to different concentrations of oestrogen and dexamethasone using MTS as previously described in section 2.4. As an initial exploratory experiment on ALP expression, cells from the osteoblastic cell line ROS 17/2.8 were tested with oestrogen concentrations from 0-10^{-7}M, and with 10^{-8}M dexamethasone as a positive control.

For measuring oestrogen stimulation using EdU incorporation and flow cytometry analysis, the cells were seeded into 6-well plates at a density of 200,000 cells per well for test plates, 1 negative EdU control and 3 oestrogen positive controls using dexamethasone at 10^{-8} M. Positive control cultures with ROS cells and cells from the human osteoblastic cell line Saos 2. At 80% confluence, the cells were serum starved overnight in Phenol Red-free DMEM with 1% FCS.

After that the cells were stimulated with oestrogen at different concentrations (0, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}M). For the pilot experiment, cells from one rat were used. In later experiments, cells from 4 rats were used and tested whereby 3 µl of each concentration of oestrogen was added in 3 ml medium (1 in 1,000). In addition, 3 µl of EdU was also added. The cells were maintained in DMEM Phenol Red-free with 1% FCS with no FGF-2 supplemented for 48 hours. After 48 hours, the cells were collected and analysed by flow cytometry for the percentage of EdU incorporated cell populations as previously described in section 2.19.

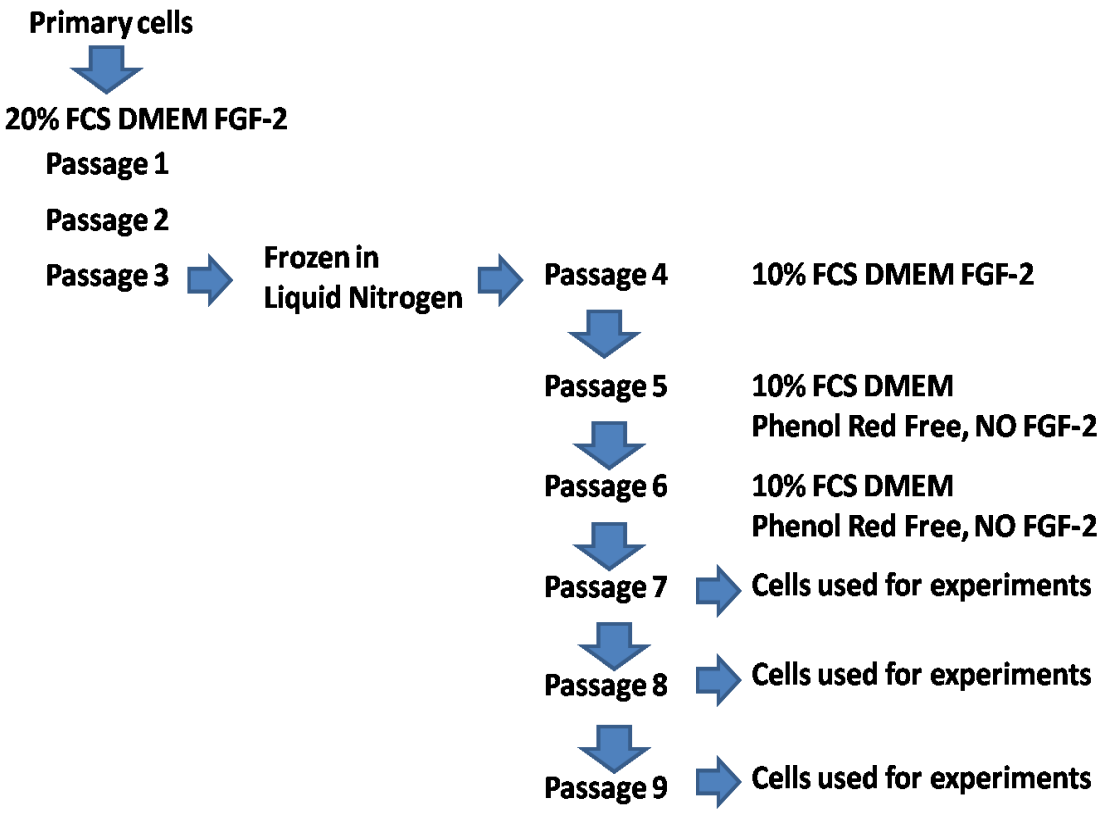


Figure 6-1: This diagram summarised how the cells were grown before they were used for oestrogen experiments.

6.3.6 Experiments using single labelling to test whether heterotypic cultures may induce changes in proliferation, total ALP activity, mineralised bone nodules, expression of transcription factors and osteoblast-associated genes or not

In these experiments cells were grown in CellTracker™ Green labelled heterotypic co-cultures and then sorted by FACS as described in Chapter 5.

6.3.6.1 Proliferation and total ALP activity in monotypic and heterotypic cultures

The cells were grown in DMEM with 10% FCS supplemented with 10 ng/ml of FGF-2. At around 80% confluence the cells were tested for proliferation by MTS assay followed by ALP total activity assay. Briefly, 2,000 cells of each cell type were sorted in 96-well plates in triplicate and were grown for periods of 7 and 14 days prior to analysis for MTS and total ALP activity in both monotypic and heterotypic cultures as previously described in section 2.4 and 2.5.

6.3.6.2 Mineralised bone nodules after grown in heterotypic culture

The cells were stained and grown together for 0 and 7 days in heterotypic cultures. Then they were sorted by cell sorter into 24-well plate at the density 100,000 cells/well. The mineralised nodule formation was induced in osteoblast differentiation medium (DMEM containing 10% FCS, no FGF-2, supplemented with 50 μg/ml of ascorbic acid, 100 nM of dexamethasone, 10 mM of β-glycerophosphate) for 28 days and stained with Alizarin red to identify bone nodules as described in section 2.9.

6.3.6.3 Transcription factors and osteoblast-associated gene expression by qRT-PCR

Similar experiments to the investigation of *Hoxa* gene expression in monotypic and heterotypic cultures described in section 5.3.4 were carried out. In these experiments, we followed the same protocol, testing for transcription factor and osteoblast-associated gene expression.

6.4 Results

6.4.1 Proliferation and ALP activity

6.4.1.1 Proliferation

The cells were grown at passage 5 and tested. There was no different in proliferation between rat calvarial and femoral osteoblastic cells from 4 rats grown in culture for 7 and 14 days (Figure 6-2).

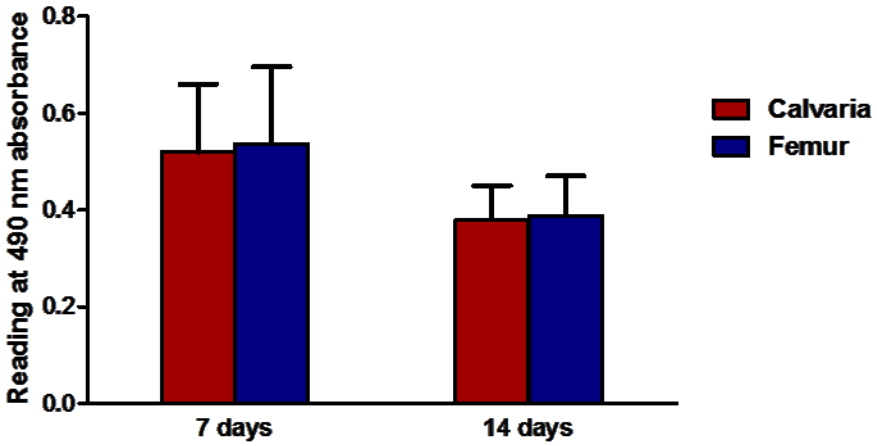


Figure 6-2: Cell proliferation of calvarial and femoral osteoblastic cells grown in culture for 7 and 14 days. Results shown as mean ± SD, 3 replicates from each of 8 different primary osteoblast cultures were used to generate these data. No difference in cell proliferation between two populations in both days. Paired *t*-test was used to compare proliferation between calvarial and femoral osteoblastic cells ($p<0.05$, considered significantly different).

6.4.1.2 ALP activity

Although the mean ALP activity was higher in femoral osteoblasts at both day 7 and day 14, this was not statistically significantly different (Figure 6-3).

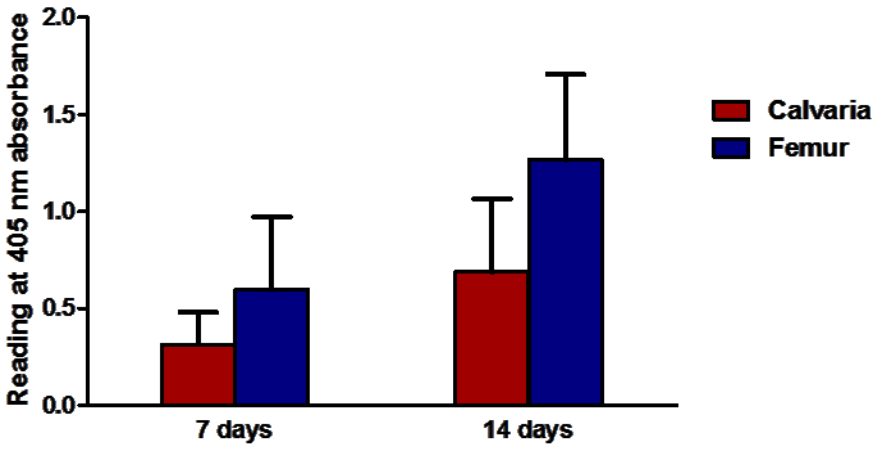


Figure 6-3: ALP activity of calvarial and femoral osteoblastic cells grown in culture for 7 and 14 days. Results shown as mean ± SD, 3 replicates from each of 8 different primary osteoblast cultures were used to generate these data. No difference in ALP activity between two populations in both days. Paired *t*-test was used to compare ALP activity between calvarial and femoral osteoblastic cells ($p<0.05$, considered significantly different).

In addition, alkaline phosphatase activity was adjusted for viable cell number using MTS assay. The data also showed the same results with no statistically significant difference in ALP activity between cells (Figure 6.4).

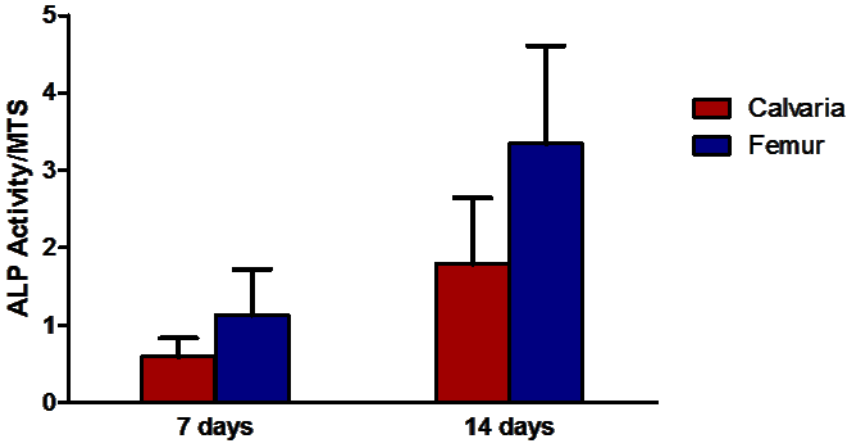


Figure 6-4: Alkaline phosphatase activity of calvarial and femoral osteoblastic cells when corrected to viable cell number using MTS assay showed total ALP activity of the cells grown in culture for 7 and 14 days. Results shown as mean ± SD, 3 replicates from each of 8 different primary osteoblast cultures were used to generate these data. No difference in total ALP activity between two populations in both days. Paired *t*-test was used to compare total ALP activity between calvarial and femoral osteoblastic cells ($p<0.05$, considered significantly different).

6.4.1.3 Additional analyses of total ALP activity

To analyse the data further and to exclude the possibility of type 2 statistical errors due to large standard deviations, the data obtained from monotypic cultures as described in Chapter 5 were used to pool the data from Day 0, Day 3 and Day 7 in monotypic culture for each culture. Consequently there were data from a total of 6 different experiments 3 different experiments for 7 days and 3 different experiments for 14 days for these analyses. Data were all normalised to ALP activity corrected to MTS of femoral cells as a percentage of that for calvarial cells and results for cells derived from each animal were analysed separately. Differences between femoral and calvarial cells were then analysed by Wilcoxon's Sign-Rank test.

In these additional analyses, there was no consistent statistically significant difference in total ALP activity in all 4 rats both in 7 days and 14 days grown in culture after passing through cells sorter as shown in Figures 6-5 a and b.

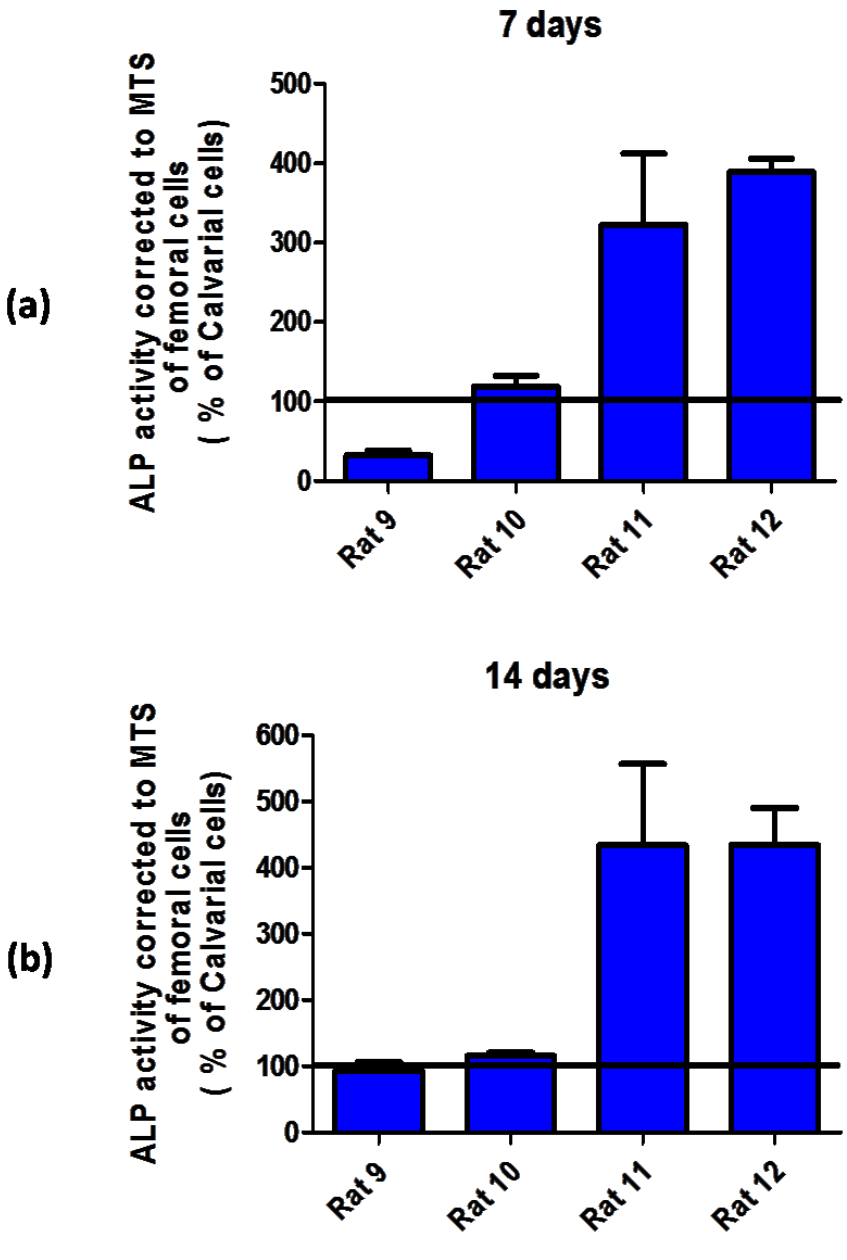


Figure 6-5: ALP activity corrected to MTS of femoral osteoblastic cells corrected to % of calvarial osteoblastic cells. Data represented as mean ± SD from 3 independent experiments for each rat matched pairs for (a) 7 days and (b) 14 days in culture after passing through cell sorter. Data were generated using 3 replicates for each culture. This showed no significant difference in total ALP activity among 4 rats. Wilcoxon's Sign-Rank test was used to compare total ALP activity between adult rat calvarial and femoral osteoblastic cells ($p<0.05$ was considered significant).

6.4.2 Osteopontin production

In order to carry out comparisons of osteopontin production between calvarial and femoral cells derived from each individual animal at p10, the data were normalised to express femoral cell osteopontin production as a percentage of that produced from calvarial osteoblastic cells. At day 10, femoral osteoblastic cells showed a borderline significant increase in osteopontin production ($p<0.06$) compared to calvarial osteoblastic cells. At Day 14 femoral osteoblastic cells also showed significantly higher production of osteopontin ($p<0.02$) although the mean size of the difference was reduced (from approximately 400% at day 10, to 200% at day 14) (Figure 6-6).

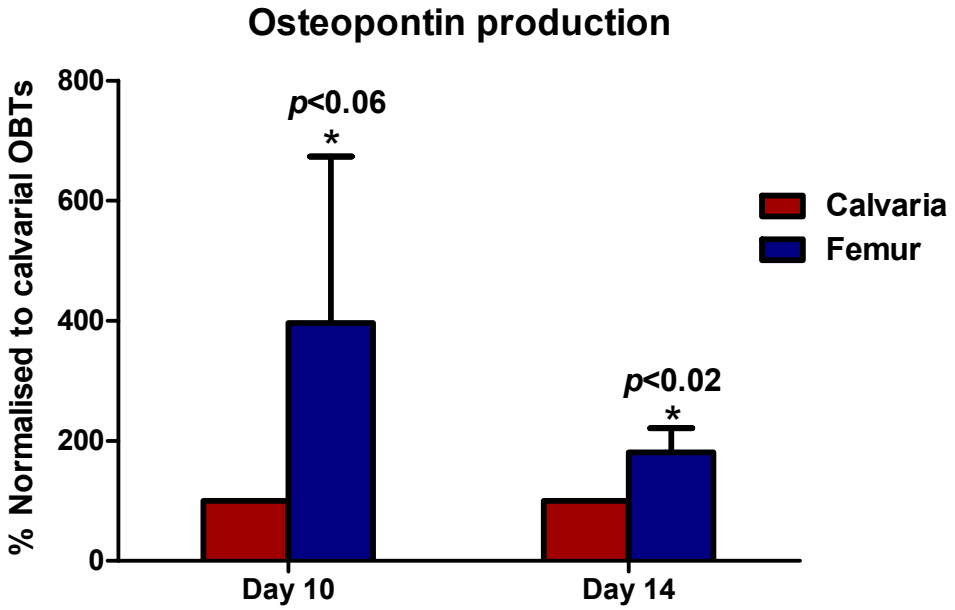


Figure 6-6: Osteopontin production detected by ELISA. Data was normalised to % of osteopontin produced from calvarial osteoblastic cells. The calvarial and femoral osteoblastic cells at p10 were grown in osteoblast culture medium supplemented with FGF-2, but without dexamethasone for 10 and 14 days. The cells were processed and collected for cell lysate. Data were generated from using 8 different primary osteoblast cultures and 2 replicates were tested for each culture. Data represented as mean ± SD. This graph shows that osteopontin can be detected in calvarial and femoral osteoblastic cells. Paired *t*-test was used to compare osteopontin production between calvarial and femoral osteoblastic cells, *, $p<0.06$ considered of borderline significance (OBTs= osteoblastic cells).

The cells at passage 10 were also tested in osteoblast culture medium supplemented with FGF-2 and 100 nM of dexamethasone for 10 days. Femoral osteoblastic cells again showed higher osteopontin production than in calvarial osteoblastic cells in all 4 rats. In addition, rat dermal fibroblasts derived from scalp, used as a negative control, had markedly lower osteopontin production than any osteoblastic cell cultures (Figure 6-7).

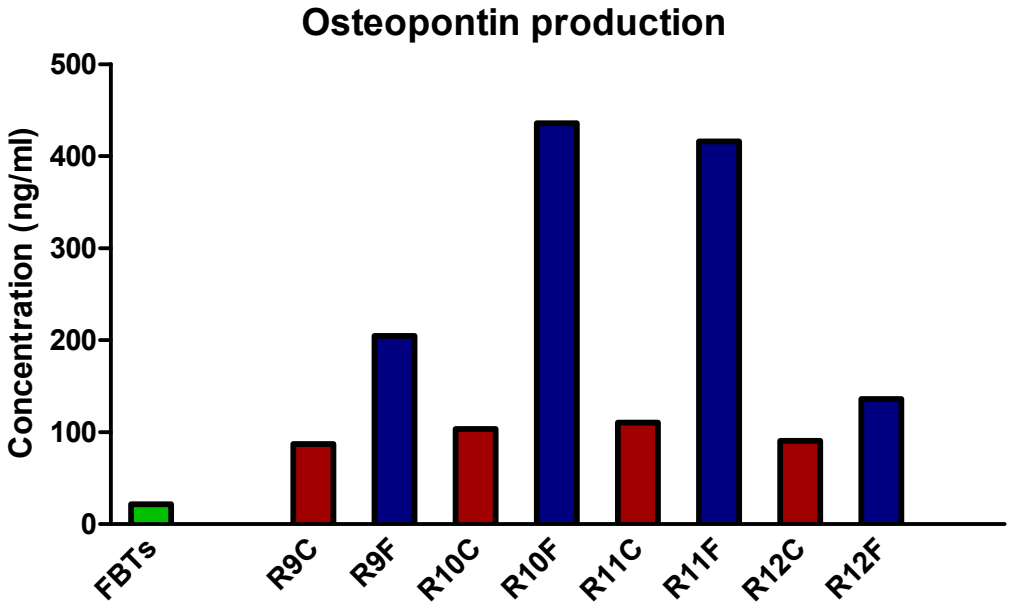


Figure 6-7: Osteopontin production (ng/ml) measured by ELISA. The calvarial and femoral osteoblastic cells were grown at passage 10 in osteoblast culture medium supplemented with FGF-2 and 100 nM of dexamethasone for 10 days. The cells were processed and collected for cell lysate. Data presented 4 different male rats (Rats 9-12), except osteopontin production in rat dermal fibroblasts derived from scalp was derived from 1 rat used as negative control. Data were generated from using 9 different primary osteoblast cultures and 2 replicates were tested for each culture. This graph shows that osteopontin can be detected in both cell types (FBTs = dermal fibroblasts derived from scalp, R9C= Rat 9 calvaria, R10C= Rat 10 calvaria, R11C= Rat 11 calvaria, R12C= Rat 12 calvaria, R9F= Rat 9 femur, R10F= Rat 10 femur, R11F= Rat 11 femur and R12F= Rat12 femur).

Again, when the data were normalised to 100% of osteopontin produced from calvarial osteoblastic cells, femoral osteoblastic cells showed significantly higher osteopontin production than calvarial osteoblastic cells (Figure 6-8).

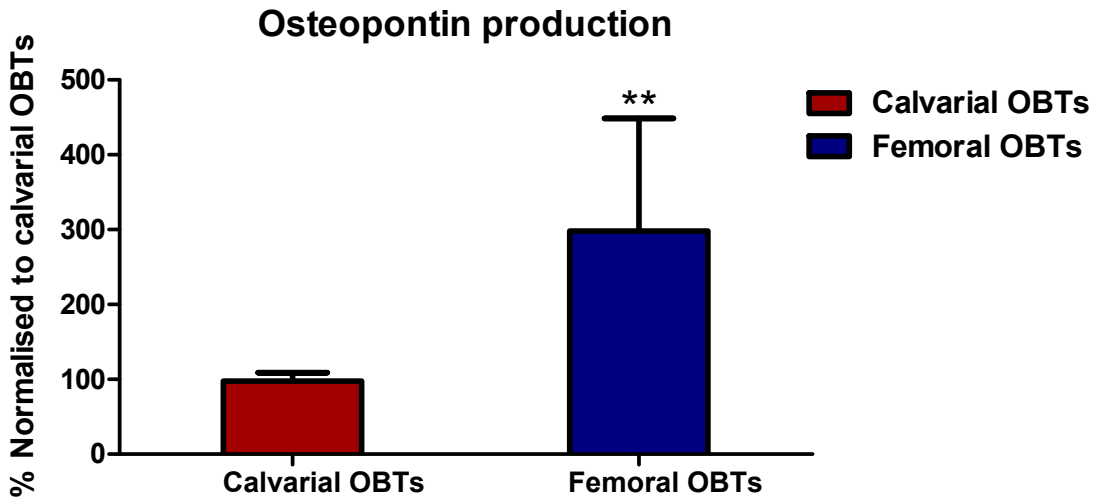


Figure 6-8: Osteopontin production detected by ELISA. Data was normalised to % of osteopontin produced from calvarial osteoblastic cells. The calvarial and femoral osteoblastic cells were grown in osteoblast culture medium supplemented with FGF-2 and 100 nM of dexamethasone for 10 days. The cells were processed and collected for cell lysate. Data were generated from using 8 different primary osteoblast cultures and 2 replicates were tested for each culture. Data represented as mean ± SD. This graph shows that osteopontin can be detected in calvarial and femoral osteoblastic cells. Paired *t*-test was used to compare osteopontin production between calvarial and femoral osteoblastic cells, **, $p<0.01$ significantly different (OBTs= osteoblastic cells).

6.4.3 Osteoblast-associated genes

The most two stable genes (*Eif4a2* and *Atp5b*) were tested from 6 House Keeping Genes (HKGs) and selected as the reference genes (see Appendix 3) to test for osteoblast-associated gene expression (*Runx2*, *Bglap* and *Spp1*) in adult rat calvarial and femoral osteoblastic cells at passage 5 and passage 10.

The calvarial and femoral osteoblastic cells were grown in osteoblast culture media supplemented with 10 ng/ml of FGF-2 and tested at passage 5. *Runx2* gene showed significantly higher expression in calvarial osteoblastic cells than in femoral osteoblastic cells ($p<0.05$) (Figure 6-9 a), whilst *Bglap* gene expression was equally expressed in both cell types (Figure 6-9 b). Mean *Spp1* expression was higher in femoral osteoblastic cells than in calvarial osteoblastic cells, but this difference did not reach statistical significance (Figure 6-9 c).

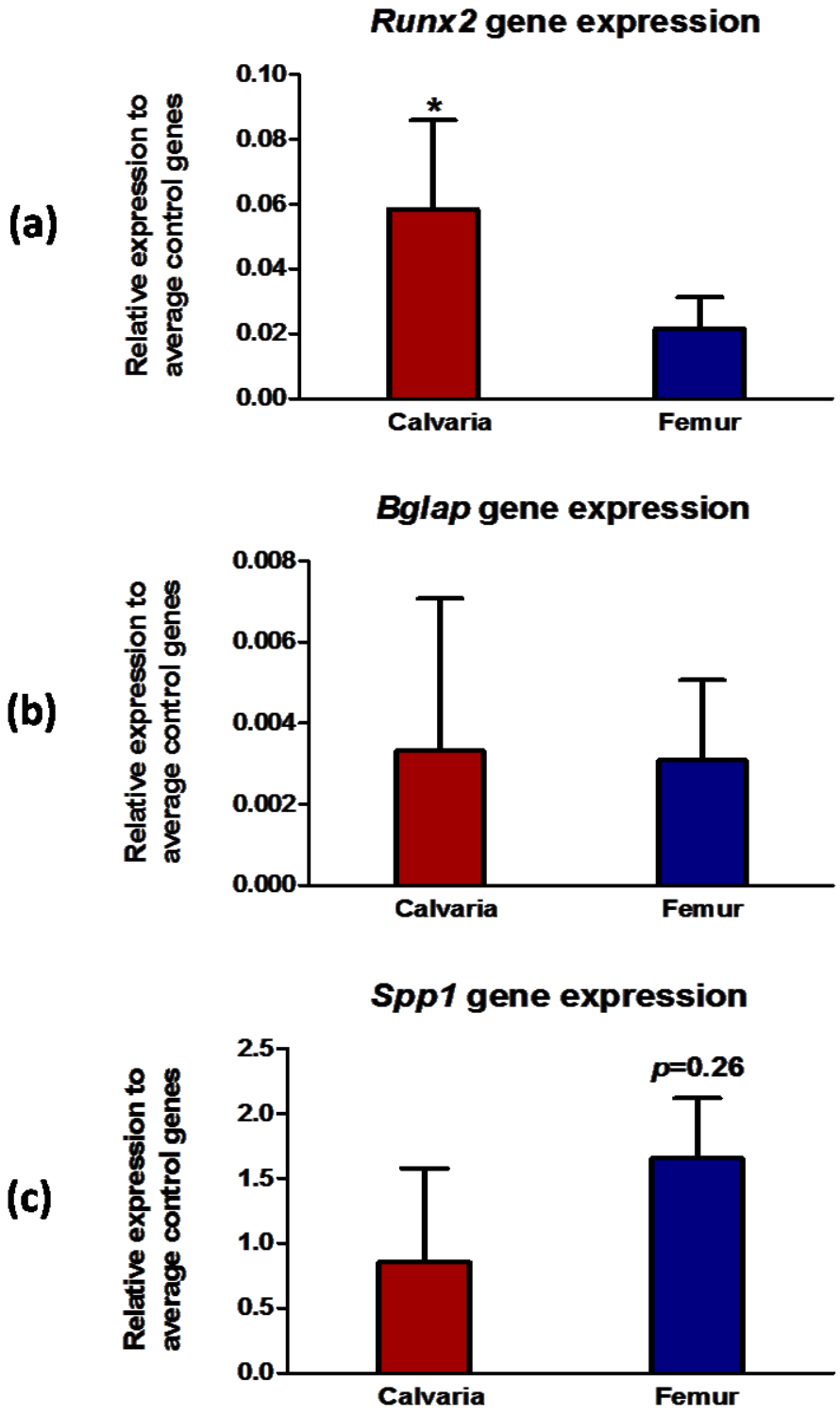


Figure 6-9: Relative expression of osteoblast-associated genes to average control genes (*Atp5b* and *Eif4a2*) in adult rat calvarial and femoral osteoblastic cells. The cells were tested at passage 5 in present of FGF-2 in culture media. Data represented as mean ± SD from 4 different male rats. Data were generated from using 8 different primary osteoblast cultures and 4 replicates were tested for each culture. Paired *t*-test was used to compare gene expression between two regionally distinct populations. (a) *Runx2* gene expressed significant higher in calvarial osteoblastic cell than in femoral osteoblastic cells (*, $p<0.05$). (b) *Bglap* gene expressed in both cell types with no significant differences ($p<0.05$). (c) *Spp1* gene expression was higher in femoral cells than in calvarial osteoblastic cells, but not statistically different (*, $p<0.05$ considered significantly different).

The two regionally distinct populations as mentioned earlier were grown in the same growth condition media supplemented with FGF-2 and were again tested at passage 10 for their osteoblast-associated gene expression.

As seen at passage 5, *Runx2* gene expression was higher in calvarial than in femoral cells, but this was not significantly different (Figure 6-10 a). However *Bglap* gene expression was significantly higher in femoral osteoblastic cells ($p<0.05$) (Figure 6-10 b). *Spp1* gene expression was higher in femoral than in calvarial osteoblastic cells, but this difference did not reach significance (Figure 6-10 c). There was no difference in relative gene expression levels between passage 5 and passage 10.

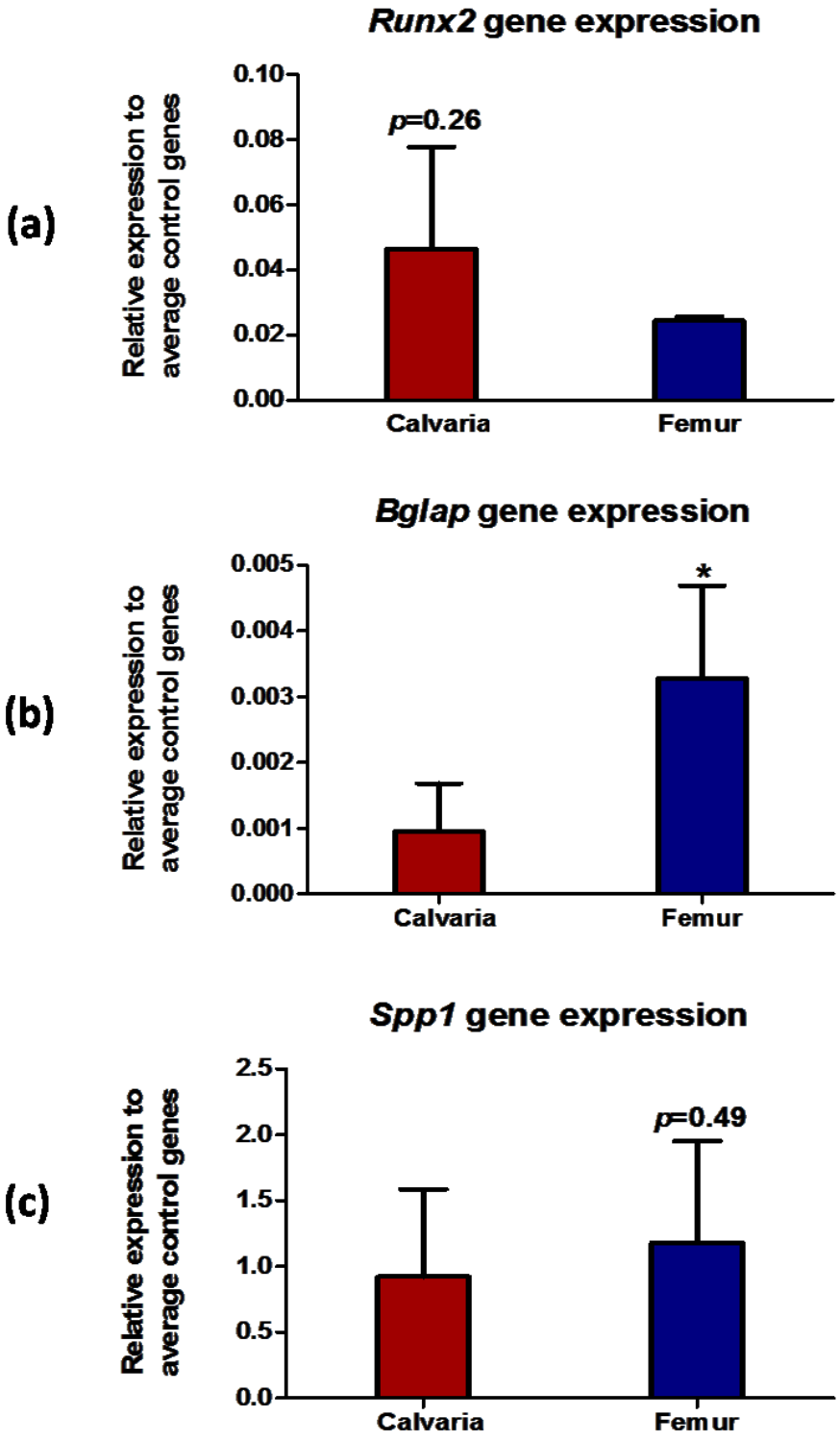


Figure 6-10: Relative expression of osteoblast-associated genes to average control genes (*Atp5b* and *Eif4a2*) in adult rat calvarial and femoral osteoblastic cells. The cells were tested at passage 10 in present of FGF-2 in culture media. Data represented as mean ± SD from 4 different male rats. Data were generated from using 8 different primary osteoblast cultures and 4 replicates were tested for each culture. Paired *t*-test was used to compare gene expression between two regionally distinct populations. (a) *Runx2* gene expressed in both cell types with no significant differences ($p<0.05$). (b) *Bglap* gene expressed significant higher in femoral osteoblastic cell than in calvarial osteoblastic cells (*, $p<0.05$). (c) *Spp1* gene expression was higher in femoral cells than in calvarial osteoblastic cells, but not statistically different (*, $p<0.05$ considered significantly different).

6.4.3.1 Additional analyses of osteoblast-associated genes

Although a number of the predicted differences in osteoblastic gene expression did not show significant differences, the "direction" of the difference in expression of specific factors between femoral and calvarial cells appeared remarkably consistent as previously described for transcription factor expression in section 4.4.3.3. Again further analyses were carried out pooling the data from p5 and p10 for each culture, and also including additional data obtained from monotypic cultures in previous experiments described in Chapter 5. Consequently, there were data from a total of 8 different experiments for these analyses. Data were all normalised to describe expression patterns of femoral cells as a percentage of that for calvarial cells and results for cells derived from each animal were analysed separately. Differences between femoral and calvarial cells were then analysed by Wilcoxon's Sign-Rank test.

In these additional analyses, *Runx2* expression was higher in calvarial osteoblastic cells than in femoral osteoblastic cells (Figure 6-11), and this was significantly different in 3 of 4 rats. *Bglap* and *Spp1* expression was higher in femoral osteoblastic cells than in calvarial osteoblastic cells (Figures 6-12 and 6-13) and these results were also statistically significantly different in 3 of 4 rats.

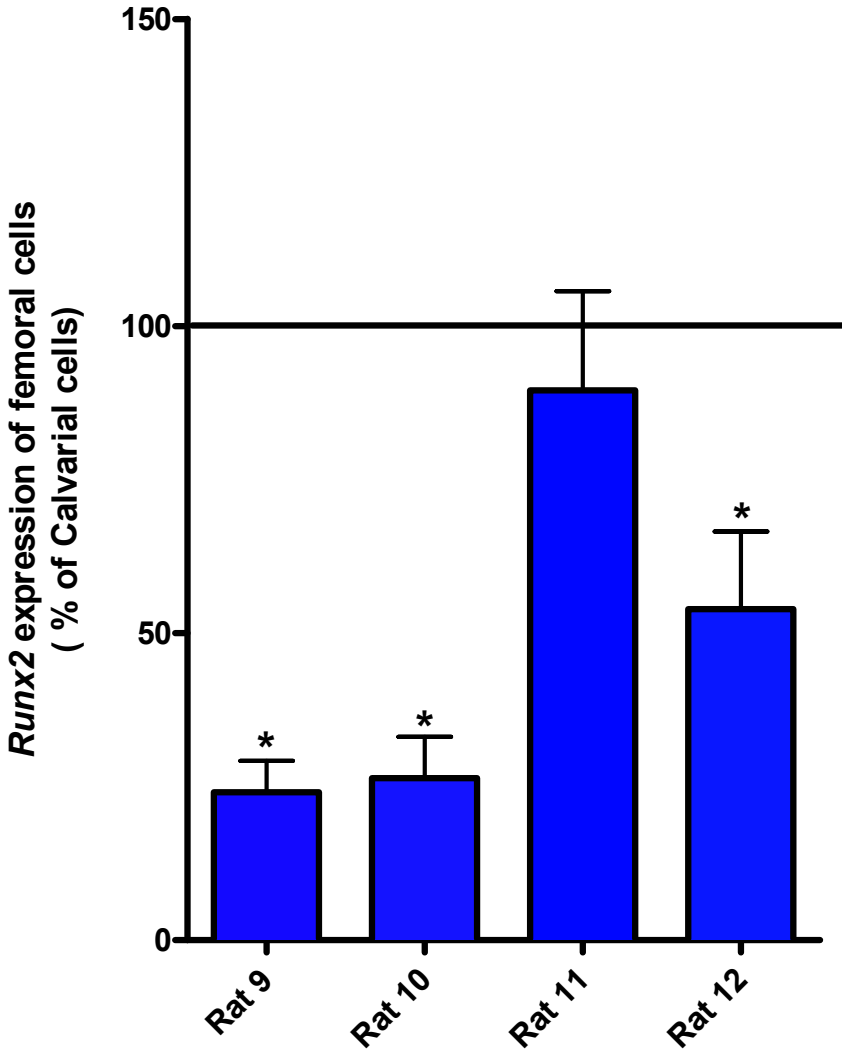


Figure 6-11: Relative expression of *Runx2* to average control genes (*Atp5b* and *Eif4a2*) of femoral osteoblastic cells corrected to % of calvarial osteoblastic cells. Data represented as mean ± SD from 8 independent experiments for each rat matched pairs. Data were generated from using 8 different primary osteoblast cultures and 4 replicates were tested for each culture. This showed *Runx2* expressed significantly higher in calvarial osteoblastic cells than in femoral osteoblastic cells. Wilcoxon's Sign-Rank test was used to compare gene expression between adult rat calvarial and femoral osteoblastic cells (*, $p<0.05$ was considered significant).

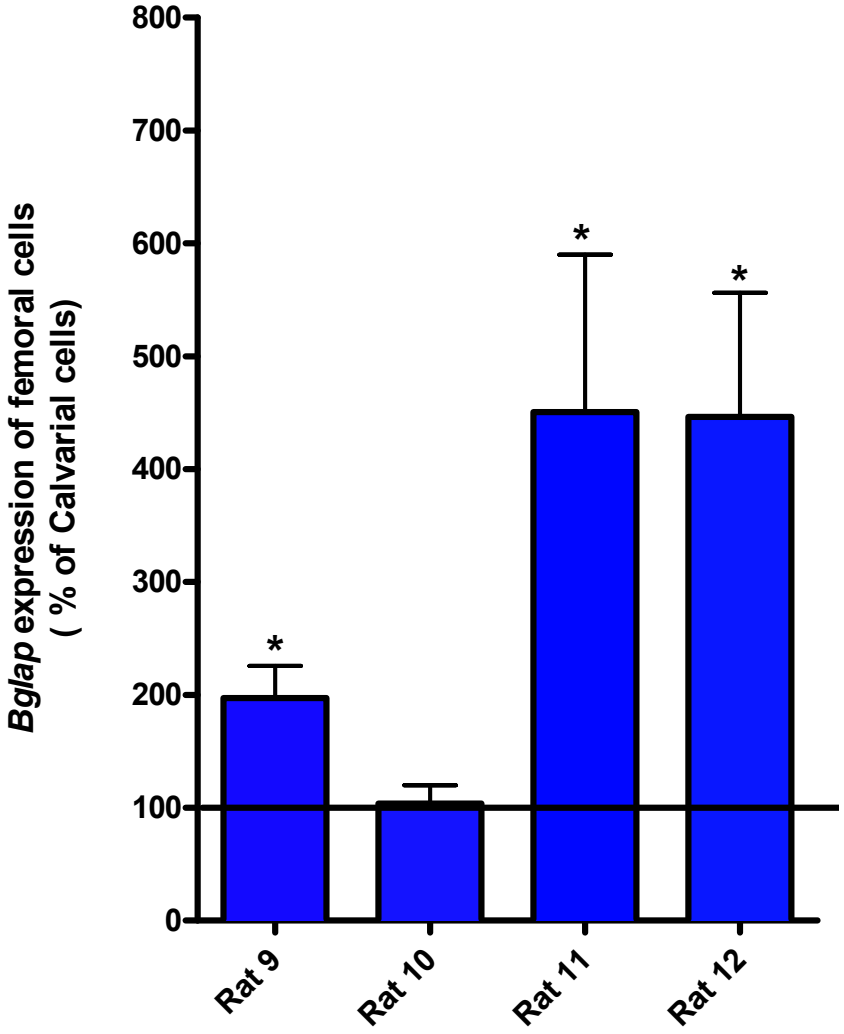


Figure 6-12: Relative expression of *Bglap* to average control genes (*Atp5b* and *Eif4a2*) of femoral osteoblastic cells corrected to % of calvarial osteoblastic cells. Data represented as mean ± SD from 8 independent experiments for each rat matched pairs. Data were generated from using 8 different primary osteoblast cultures and 4 replicates were tested for each culture. This showed *Bglap* expressed significantly higher in femoral osteoblastic cells than in calvarial osteoblastic cells. Wilcoxon's Sign-Rank test was used to compare gene expression between adult rat calvarial and femoral osteoblastic cells (*, $p<0.05$ was considered significant).

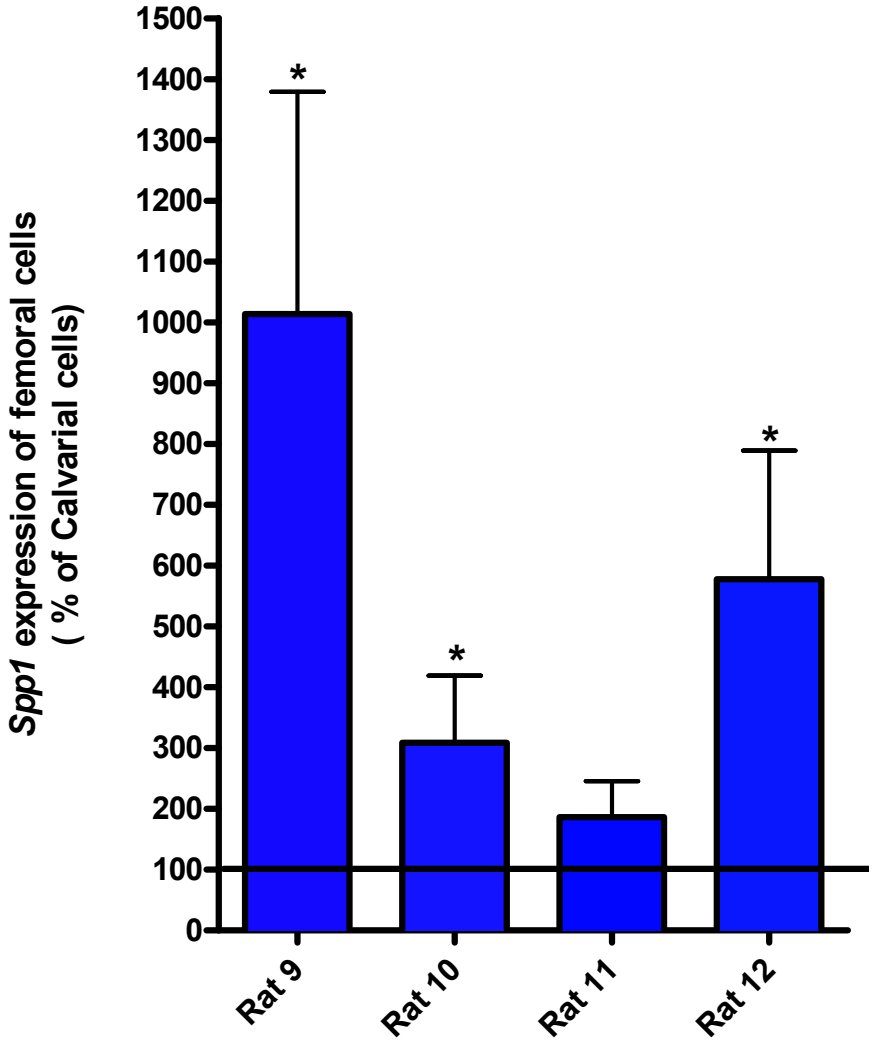


Figure 6-13: Relative expression of *Spp1* to average control genes (*Atp5b* and *Eif4a2*) of femoral osteoblastic cells corrected to % of calvarial osteoblastic cells. Data represented as mean ± SD from 8 independent experiments for each rat matched pairs. Data were generated from using 8 different primary osteoblast cultures and 4 replicates were tested for each culture. This showed *Spp1* expressed significantly higher in femoral osteoblastic cells than in calvarial osteoblastic cells. Wilcoxon's Sign-Rank test was used to compare gene expression between adult rat calvarial and femoral osteoblastic cells (*, $p<0.05$ was considered significant).

6.4.4 Oestrogen stimulation

6.4.4.1 Assessment of metabolic activity using MTS

When cells were stimulated with different concentrations of oestrogen (0, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} M) and dexamethasone 10^{-8} M, there were no differences seen in all oestrogen stimulations compared to controls either at day 3 or day 7 (Figures 6-14 and 6-15).

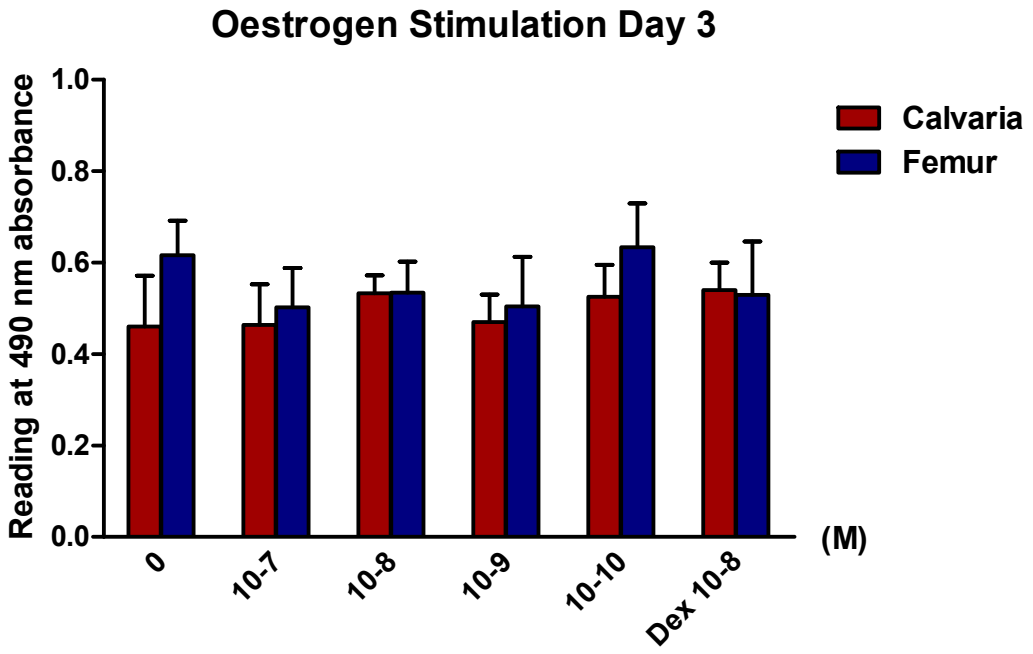


Figure 6-14: Oestrogen stimulation for 3 days with different concentration (0, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} M) and dexamethasone 10^{-8} M on calvarial and femoral osteoblastic cells after serum starving. The MTS assessed for proliferation after oestrogen stimulation in calvarial and femoral osteoblastic cells was no different in all stimulations compared to control, each concentration using 3 replicates.

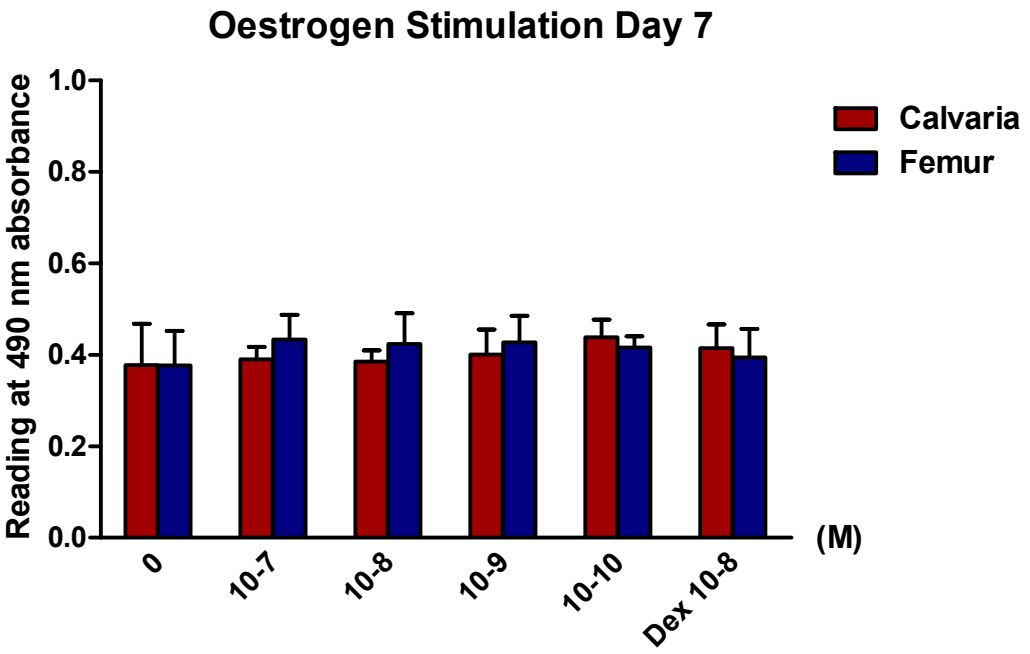


Figure 6-15: Oestrogen stimulation for 7 days with different concentration (0, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} M) and dexamethasone 10^{-8} M on calvarial and femoral osteoblastic cells after serum starving. The MTS assessed for proliferation after oestrogen stimulation in calvarial and femoral osteoblastic cells was no different in all stimulations compared to control (mean ± SD), each concentration using 3 replicates.

6.4.4.2 Assessment of ALP activity using ROS cells

When ROS cells were stimulated with different concentrations of oestrogen (0, 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11}, 10^{-12} M) and dexamethasone 10^{-8} M for 7 days, there were no differences seen in all oestrogen stimulations compared to controls. However, ROS cells showed high level of ALP activity when stimulated with dexamethasone 10^{-8} M (Figure 6-16).

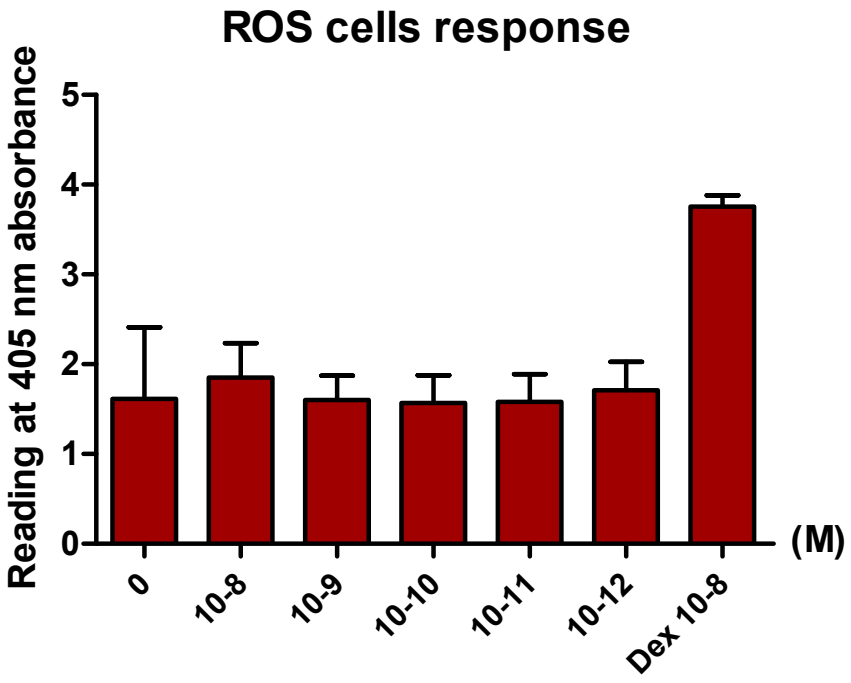


Figure 6-16: Oestrogen stimulation for 7 days with different concentration (0, 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11}, 10^{-12} M) and dexamethasone 10^{-8} M on ROS cells. The ALP activity was assessed. ROS cells showed high level of ALP activity when stimulated with dexamethasone 10^{-8} M. There was no different in all oestrogen stimulations compared to controls (mean ± SD), each concentration using 3 replicates.

6.4.4.3 EdU labelling

a) Pilot study in 1 rat

The results of the initial pilot study on calvarial and femoral osteoblastic cells from one rat (Rat 9) are shown in Figures 6-17 and 6-18. Unstimulated control cultures showed no significant EdU labelling. However stimulation with oestrogen resulted in labelling of about 10% of cells, although there were no differences between calvarial and femoral osteoblastic cells except at 10^{-10}M oestrogen, and no evidence of a dose-response across different concentrations of oestrogen.

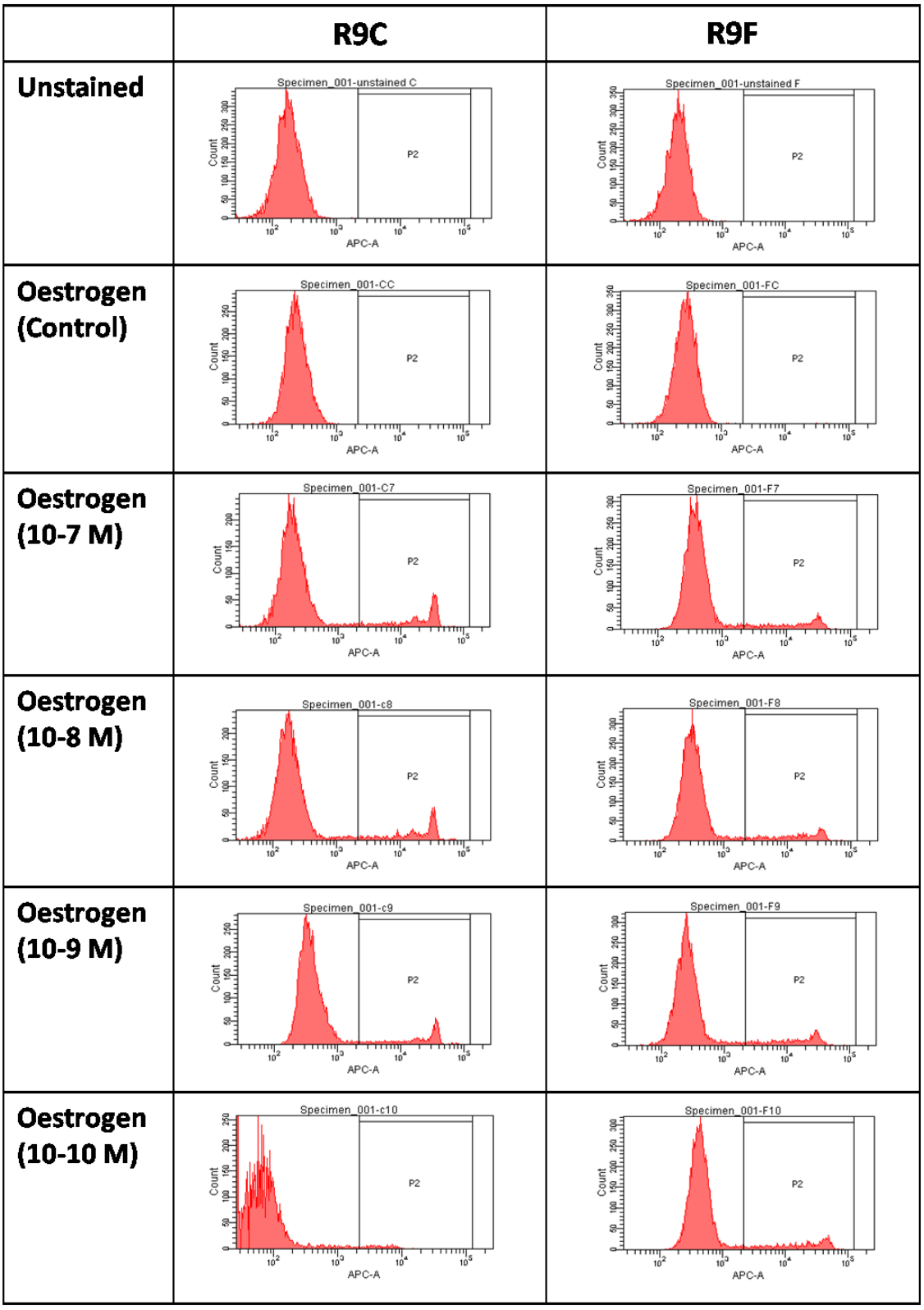


Figure 6-17: Pilot data of oestrogen respond on calvarial and femoral osteoblastic cells from one male rat (Rat 9) stimulated with different concentration of oestrogen (0, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} M). After 48 hours, EdU incorporated cells were analysed by flow cytometry.

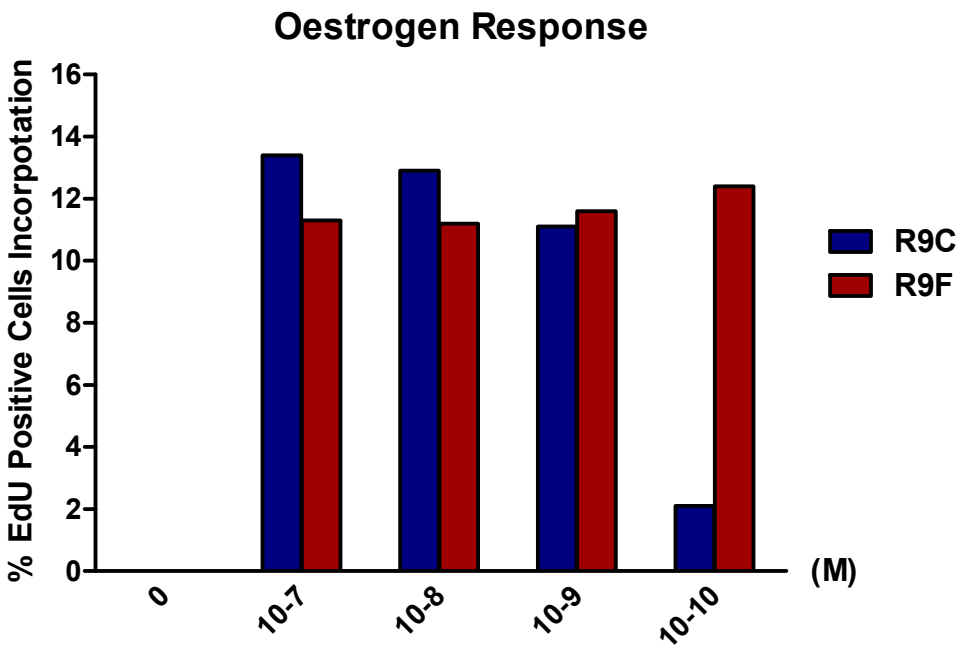


Figure 6-18: Graphical summary of pilot data of oestrogen stimulation with different concentration (0, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} M) on calvarial and femoral osteoblastic cells from one male rat (Rat 9).

b) Further experiments with cells from 4 animals

Further definitive experiments were carried out as described in the pilot experiment, using the calvarial and femoral osteoblastic cells from 4 rats. ROS cells and Saos2 cell lines were used as positive controls. There was no evidence of oestrogen response in ROS cells or Saos2 cell lines compared to controls (Figure 6-19). There was also no evidence in oestrogen response in both osteoblastic cells (Figures 6-20 and 6-21), unlike in the previous pilot experiment.

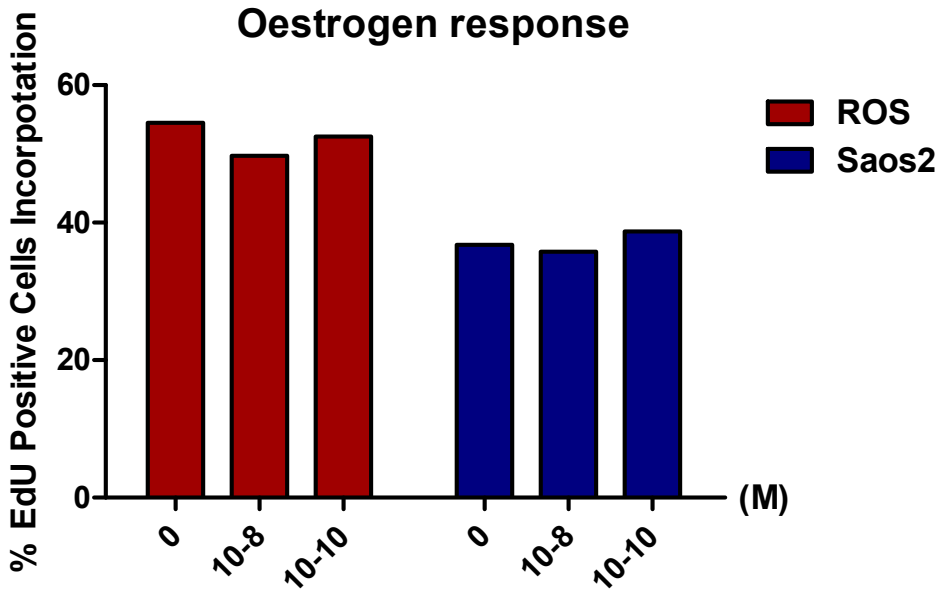


Figure 6-19: Oestrogen stimulation with different concentration (0, 10^{-8}, 10^{-10} M) on ROS cells and Saos2 cell lines. No difference in oestrogen responsiveness was observed.

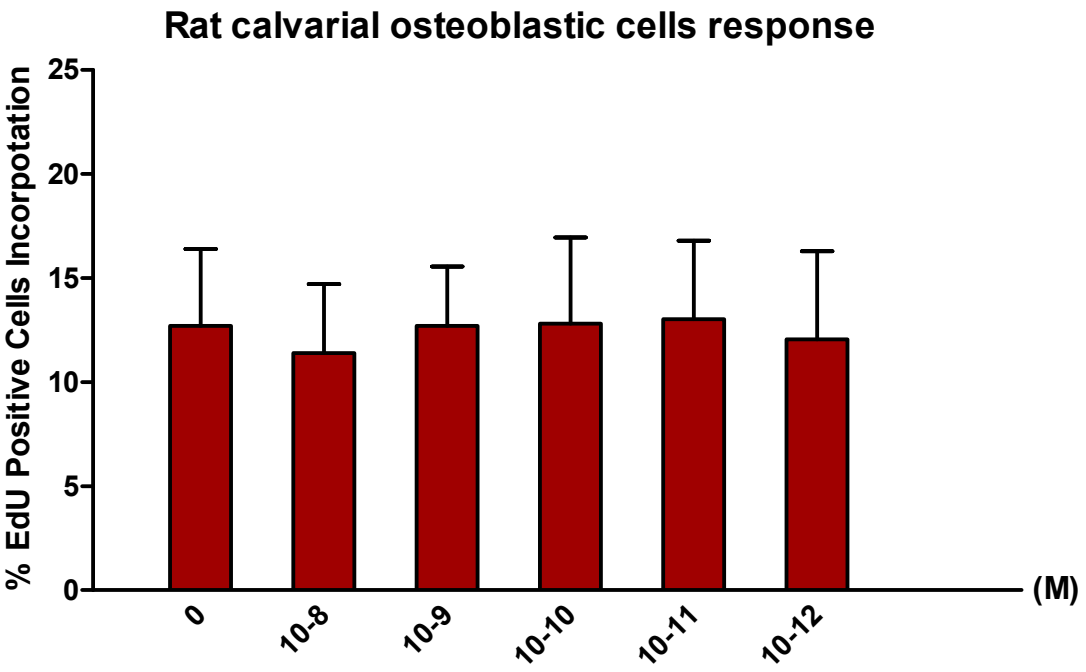


Figure 6-20: Oestrogen stimulation with different concentration (0, 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11}, 10^{-12} M) on calvarial osteoblastic cells. No difference in oestrogen response was observed (mean ± SD).

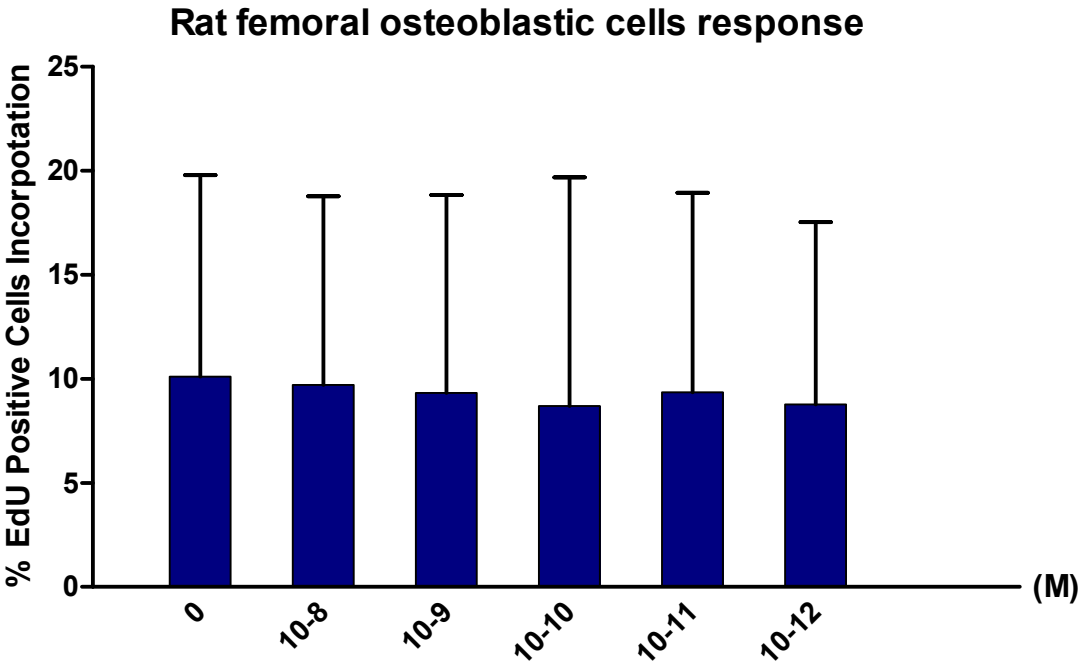


Figure 6-21: Oestrogen stimulation with different concentration (0, 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11}, 10^{-12} M) on femoral osteoblastic cells. No difference in oestrogen response was evidenced (mean ± SD).

6.4.5 Experiments using single labelling show that heterotypic cultures did not induce changes in proliferation, total ALP activity, mineralised bone nodules, expression of transcription factors and osteoblast-associated genes

6.4.5.1 Proliferation

At Day 0, Day 3 and Day 7 in monotypic culture, there was no different in proliferation between rat calvarial and femoral osteoblastic cells after passing through the cell sorter and continuing to grow for 7 and 14 days (Figure 6-22).

In heterotypic culture, there was no significant difference between the two distinct cell populations. In addition, when looking at days in cultures after cell sorting, both osteoblastic cells showed no significant differences (Figure 6-23).

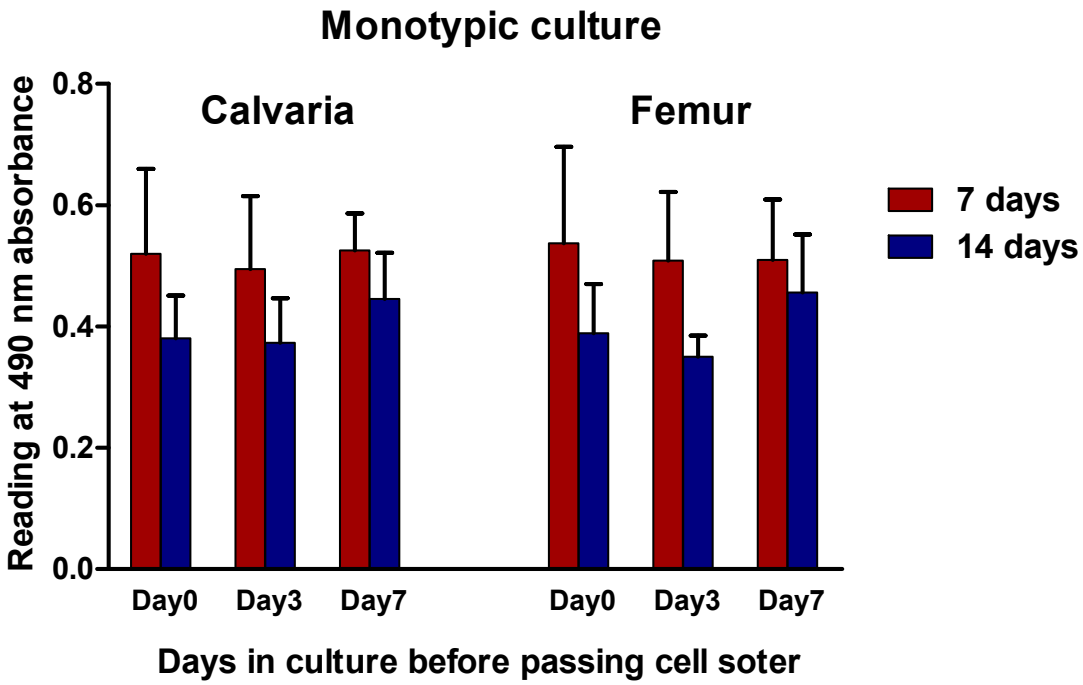


Figure 6-22: Cell proliferation of calvarial and femoral osteoblastic cells in monotypic culture at Day 0, Day 3 and Day 7 after passing through cell sorter and continuing to grow in culture for 7 and 14 days. Results shown as mean ± SD, each sample using 3 replicates. No difference in cell proliferation between two populations. Paired *t*-test was used to compare proliferation between calvarial and femoral osteoblastic cells ($p<0.05$, considered significantly different).

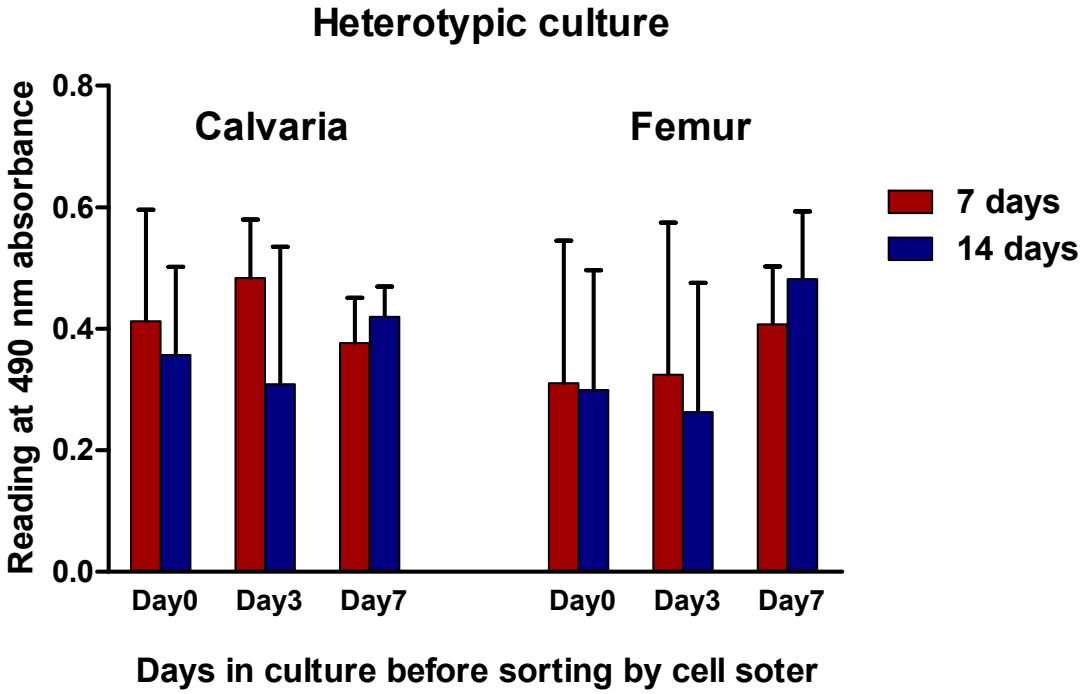


Figure 6-23: Cell proliferation of calvarial and femoral osteoblastic cells grown in heterotypic culture at Day 0, Day 3 and Day 7 and continued to grow in culture after sorting for 7 and 14 days. Results shown as mean ± SD, each sample using 3 replicates. No difference in cell proliferation between two populations. Paired *t*-test was used to compare proliferation between calvarial and femoral osteoblastic cells ($p<0.05$, considered significantly different).

6.4.5.2 ALP activity

At Day 0, Day 3 and Day 7 in monotypic culture, there was no difference in ALP activity between rat calvarial and femoral osteoblastic cells after passing through the cell sorter and continuing to grow for 7 and 14 days (Figure 6-24).

In heterotypic culture, there was no significant difference between the two distinct cell populations. In addition, when looking at days in cultures after cell sorting, both osteoblastic cells also showed no significant differences (Figure 6-25).

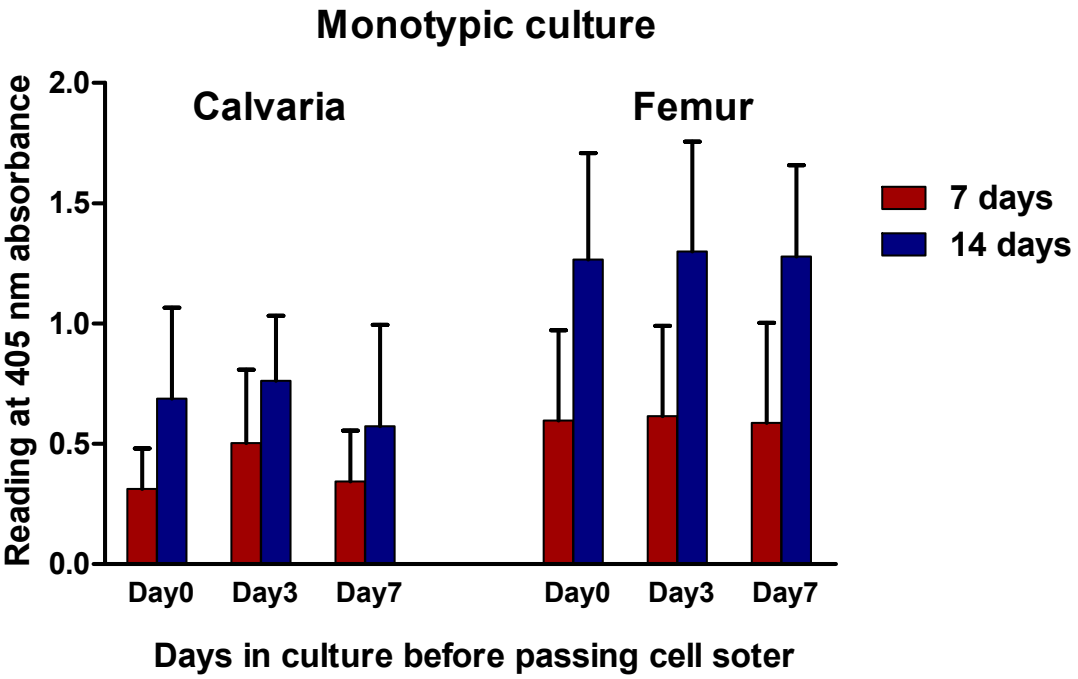


Figure 6-24: ALP activity of calvarial and femoral osteoblastic cells in monotypic culture at Day 0, Day 3 and Day 7 after passing through cell sorter and continuing to grow in culture for 7 and 14 days. Results shown as mean ± SD, each sample using 3 replicates. No difference in ALP activity between two populations. Paired *t*-test was used to compare proliferation between calvarial and femoral osteoblastic cells ($p<0.05$, considered significantly different).

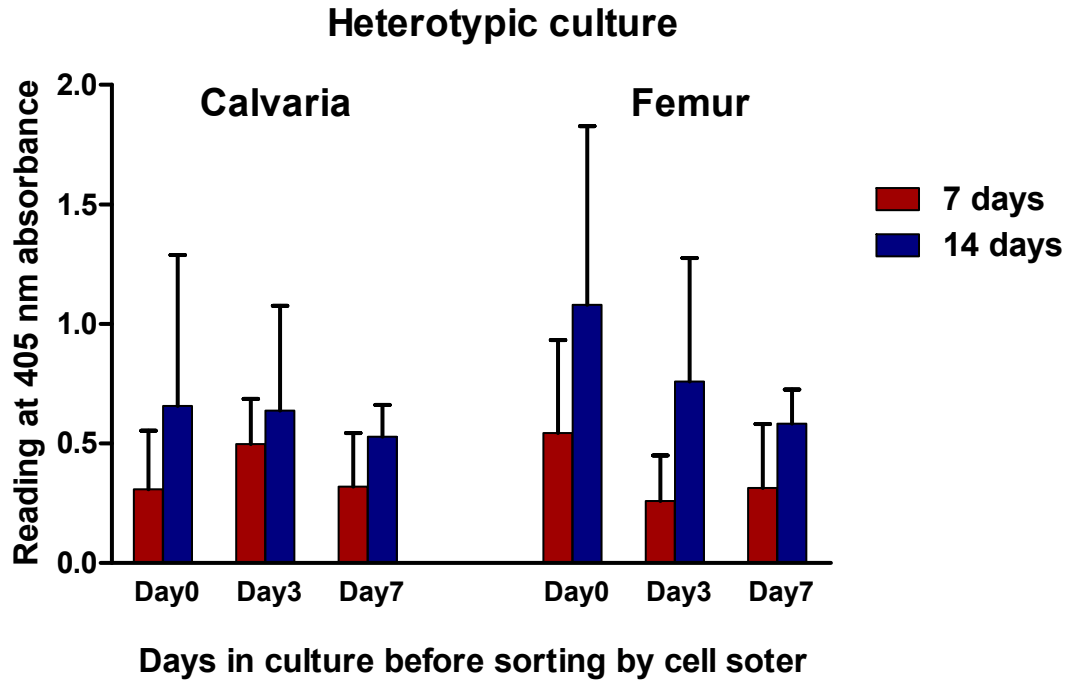


Figure 6-25: ALP activity of calvarial and femoral osteoblastic cells grown in heterotypic cultures at Day 0, Day 3 and Day 7 and continued to grow separately in culture after sorting for 7 and 14 days. Results shown as mean ± SD, each sample using 3 replicates. No difference in ALP activity between two populations. Paired *t*-test was used to compare proliferation between calvarial and femoral osteoblastic cells ($p<0.05$, considered significantly different).

6.4.5.3 Total ALP activity corrected for MTS

Alkaline phosphatase activity of calvarial and femoral osteoblastic cells was adjusted for viable cell number. The data showed the same results as before. Total ALP activity when cells were grown in culture after passing through cell sorter showed similar levels between two populations in monotypic culture (Figure 6-26).

In heterotypic culture, there was no significant difference between the two distinct cell populations. In addition, when looking at days in cultures after cell sorting, both groups of osteoblastic cells also showed no significant differences (Figure 6-27).

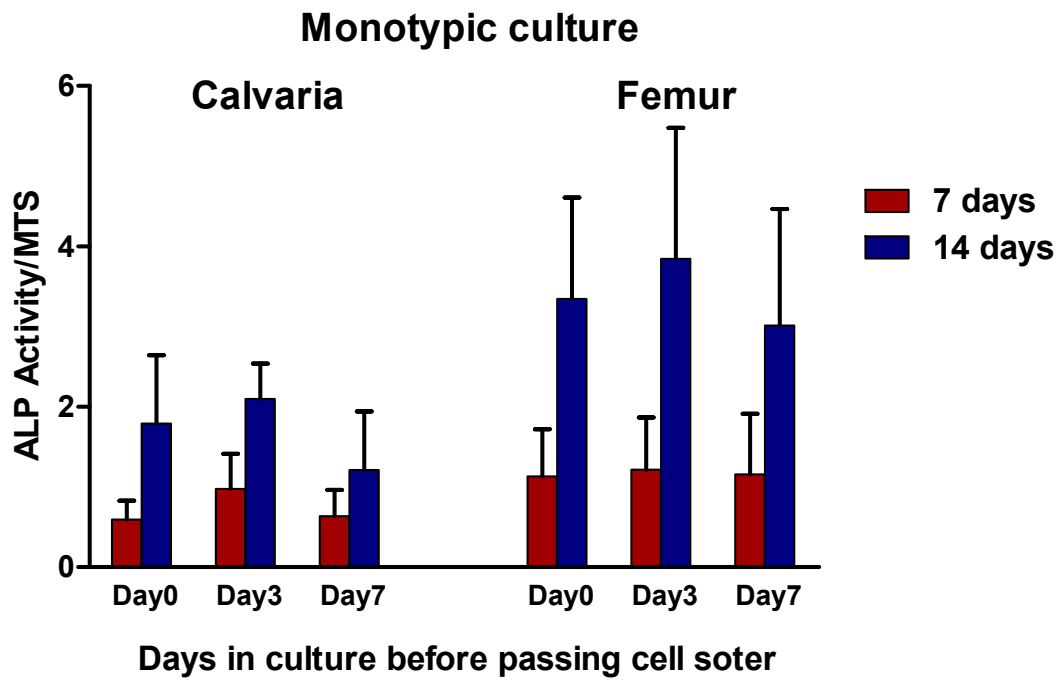


Figure 6-26: Alkaline phosphatase activity of calvarial and femoral osteoblastic cells when corrected to viable cell number using MTS assay. Two different populations were grown at Day 0, Day 3 and Day 7 in monotypic culture after passing through cell sorter and continuing to grow in culture for 7 and 14 days. Results shown as mean ± SD, each sample using 3 replicates. No difference in total ALP activity in this monotypic culture. Paired *t*-test was used to compare proliferation between calvarial and femoral osteoblastic cells ($p<0.05$, considered significantly different).

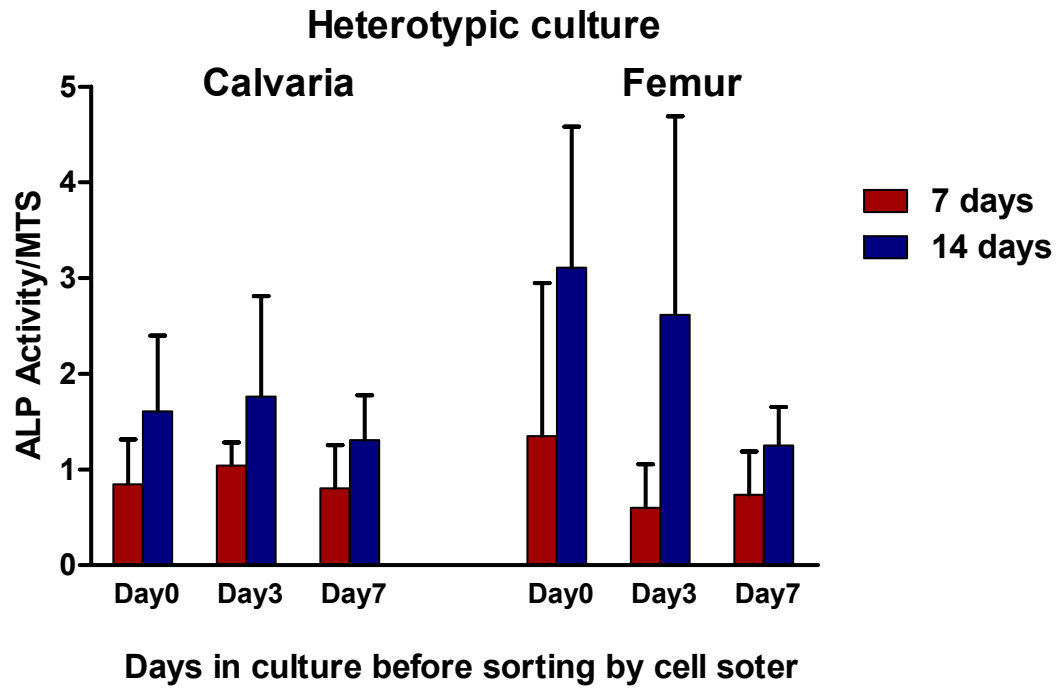


Figure 6-27: Alkaline phosphatase activity of calvarial and femoral osteoblastic cells when corrected to viable cell number using MTS assay. Two different populations were grown in heterotypic cultures at Day 0, Day 3 and Day 7 and continued to grow separately in culture after sorting for 7 and 14 days. Results shown as mean ± SD, each sample using 3 replicates. No difference in total ALP activity in this heterotypic culture. Paired *t*-test was used to compare proliferation between calvarial and femoral osteoblastic cells ($p<0.05$, considered significantly different).

6.4.5.4 Mineralised bone nodules after heterotypic cultures

To test the production of mineralised bone nodules by calvarial and femoral osteoblastic cells when grown together in heterotypic culture. Cells were grown together in heterotypic culture for 0 day as a control group and 7 days as a test group as previously described. The cells were sorted to separate two distinct populations into 24-well plates and then grown separately in osteoblast differentiation media with β-glycerophosphate for 28 days before Alizarin red staining for mineralised bone nodules. All calvarial osteoblastic cells formed fewer and smaller bone nodules than femoral osteoblastic cells in all cultures at both day 0 and day 7 of co-culture (Figures 6-28 and 6-29). There was no evidence of increased bone nodule formation in calvarial cells from day 0 to day 7 in co-cultures.

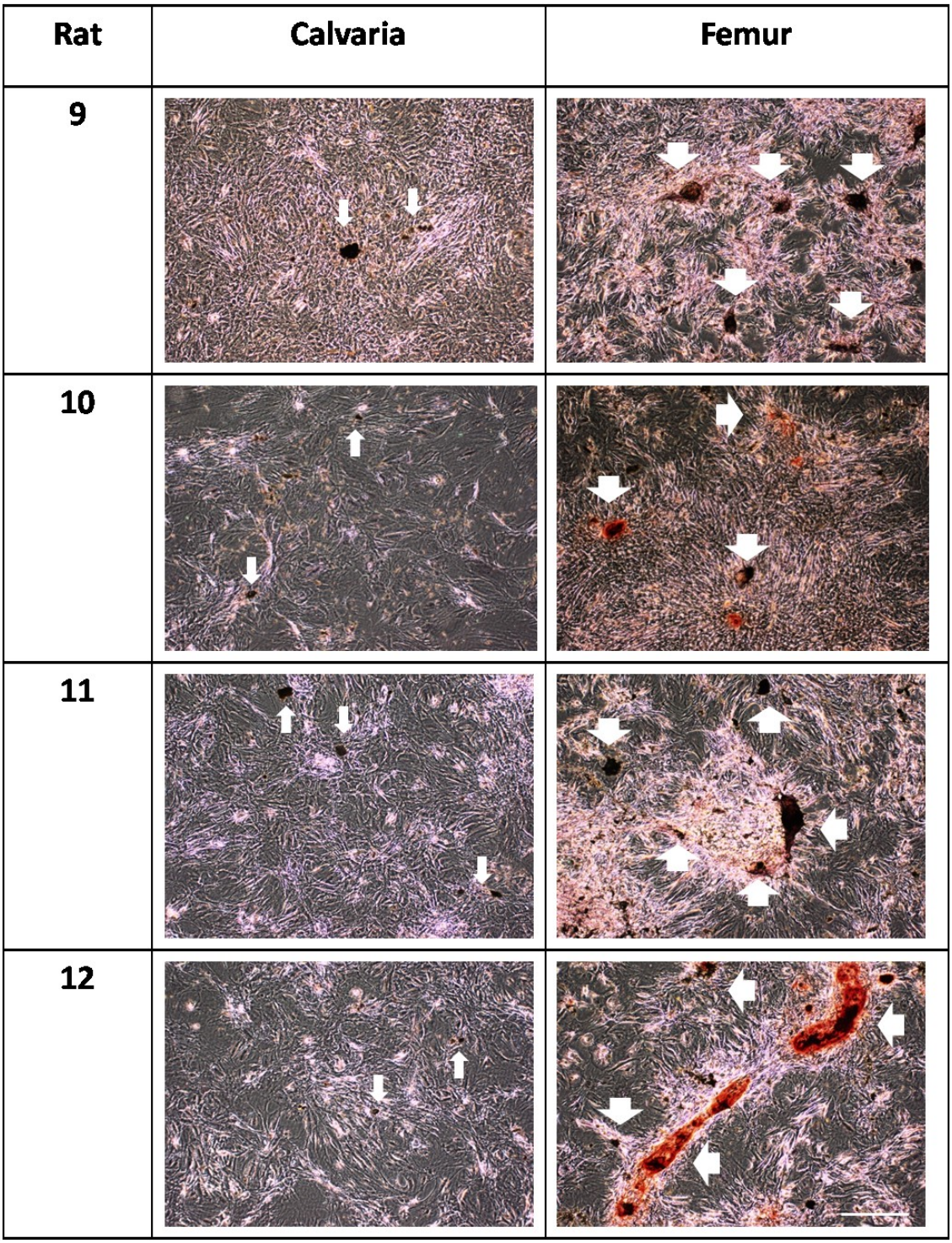


Figure 6-28: Rat calvarial and femoral osteoblastic cells from 4 male rats (Rats 9-12) were grown in heterotypic culture for 0 day as a control group and sorted by cell sorter and continued to grow separately for 28 days in osteoblast differentiation media with dexamethasone and β-glycerophosphate, but no FGF-2 supplemented before Alizarin red staining for mineralised bone nodules (arrows). All calvarial osteoblastic cells presented less bone nodules (small arrows) than femoral osteoblastic cells in all cultures at 0 day grown in heterotypic culture, at magnification 100, scale bar = 200 μm.

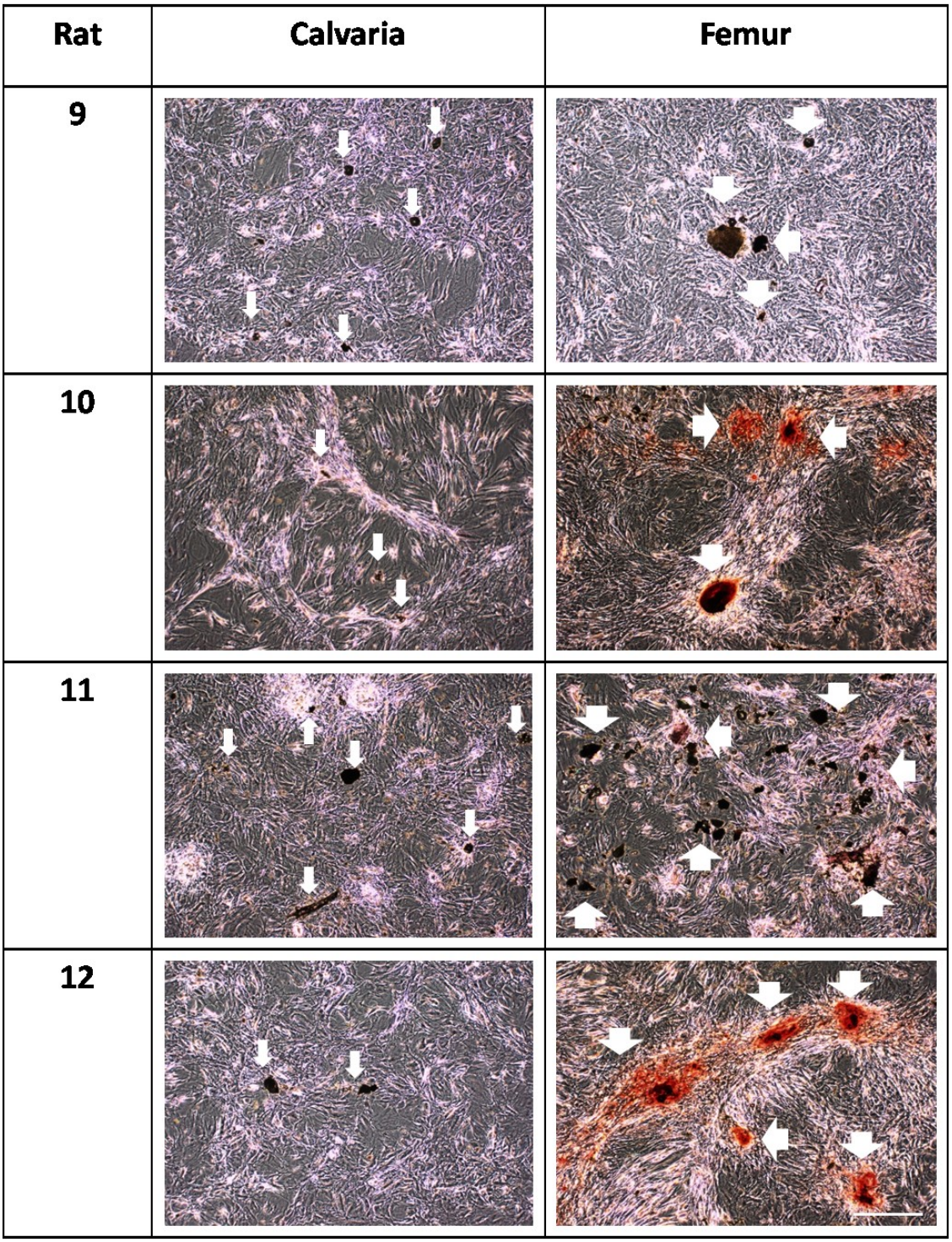


Figure 6-29: Rat calvarial and femoral osteoblastic cells from 4 male rats (Rats 9-12) were grown in heterotypic culture for 7 days as a test group and sorted by cell sorter and continued to grow separately for 28 days in osteoblast differentiation media with dexamethasone and β-glycerophosphate, but no FGF-2 supplemented before Alizarin red staining for mineralised bone nodules (arrows). All calvarial osteoblastic cells presented less bone nodules (small arrows) than femoral osteoblastic cells in all cultures at 7 days grown in culture, magnification 100, scale bar = 200 μm.

6.4.5.5 Transcription factors and osteoblast-associated gene expression

We set up labelled monotypic and heterotypic cultures as previously described in Chapter 5.

a) Calvarial osteoblastic cells stained with CellTracker™ Green

As expected gene expression did not change in monotypic cultures. In monotypic cultures, rat calvarial osteoblastic cells were stained with CellTracker™ Green, whilst femoral osteoblastic cells were unstained. The cells were passed through a cell sorter, grown and collected for RNA extraction, cDNA synthesis and qRT-PCR.

Relative expression of transcription factors (*Msx2*, *Irx5* and *Tbx3*) and osteoblast-associated genes (*Runx2*, *Bglap* and *Spp1*) to average control genes (*Atp5b* and *Eif4a2*) was assessed. Data generated from 4 different male rats expressed as mean ± SD.

Msx2 and *Irx5* were preferentially expressed in calvarial osteoblastic cells (Figure 6-30 a and b), whilst *Tbx3* was preferentially expressed in femoral osteoblastic cells (Figure 6-30 c) from Day 0 to Day 7 in all cultures. At Day 0, *Irx5* expression in calvarial osteoblastic cells was significantly greater than expression in femoral osteoblastic cells ($p<0.01$). At Day 3 and Day 7, *Irx5* expression in calvarial osteoblastic cells was also significantly higher than in femoral osteoblastic cells ($p<0.05$).

On the other hand, at Day 3 *Tbx3* expression in femoral osteoblastic cells was significantly higher than in calvarial osteoblastic cells ($p<0.01$). A similar pattern was seen at Day 7 ($p<0.05$).

From Day 0 to Day 7, *Runx2* expression was higher in calvarial osteoblastic cells and significantly different at Day 3 ($p<0.05$) (Figure 6-31 a), whilst *Bglap* and *Spp1* expression was not significantly different (Figure 6-31 b and c) from Day 0 to Day 7.

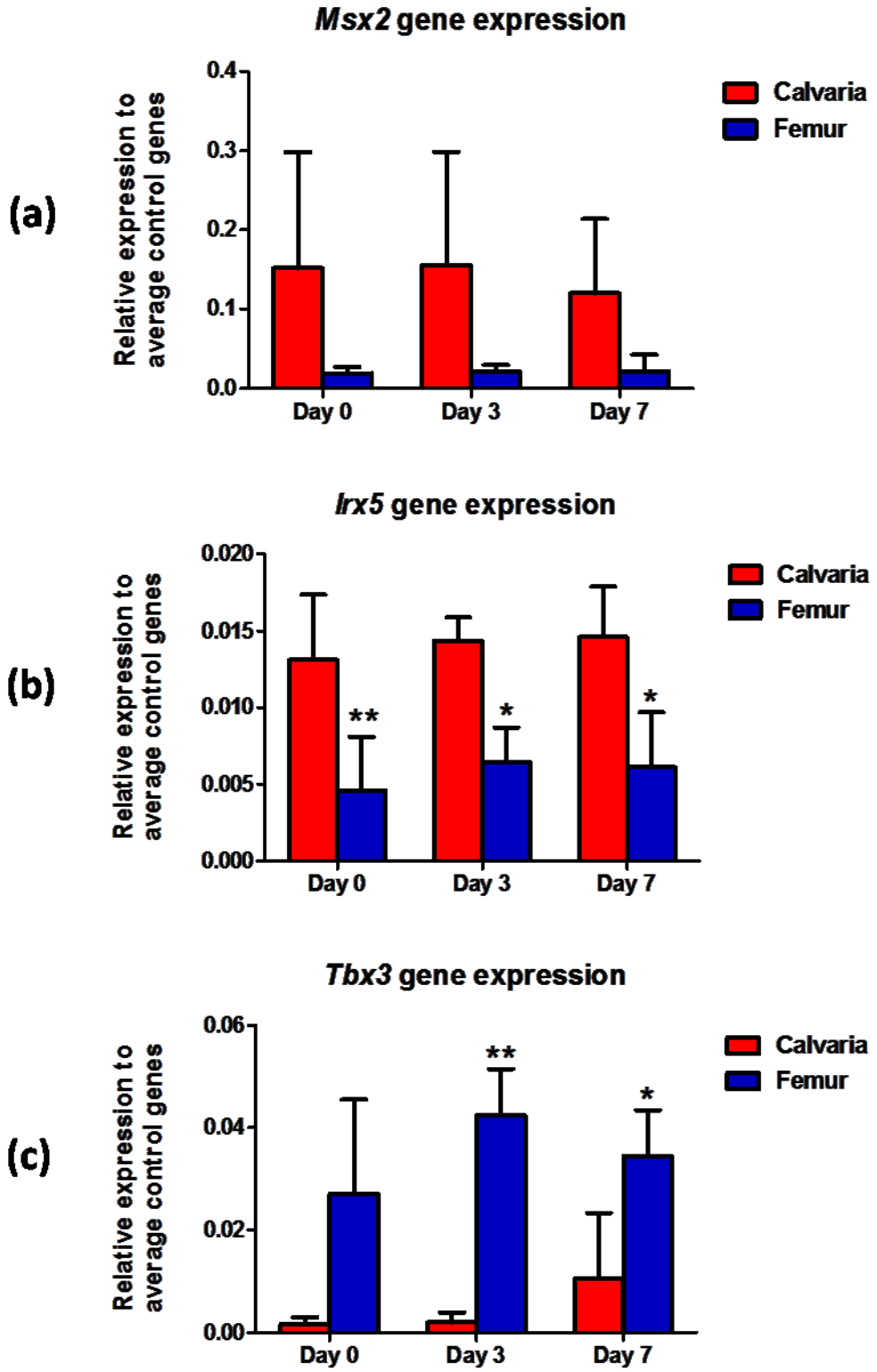


Figure 6-30: Relative expression of *Msx2, Irx5* and *Tbx3* to average control genes (*Atp5b* and *Eif4a2*). In monotypic culture, calvarial osteoblastic cells stained with CellTracker™ Green, whilst femoral osteoblastic cells were unstained. At Day 0, Day 3 and Day7, the cells passed through cell sorter, grown and collected for RNA extraction, cDNA synthesis and qRT-PCR. Data represented as mean ± SD from 4 different male rats. Data were generated from using 8 different primary osteoblast cultures and 4 replicates were tested for each culture. *Msx2* and *Irx5* were preferentially expressed in calvarial osteoblastic cells (a) and (b), whilst *Tbx3* were preferentially expressed in femoral osteoblastic cells (c). Paired *t*-test was used to compare gene expression between calvarial and femoral osteoblastic cells, *, $p<0.05$, **, $p<0.01$ considered significantly different.

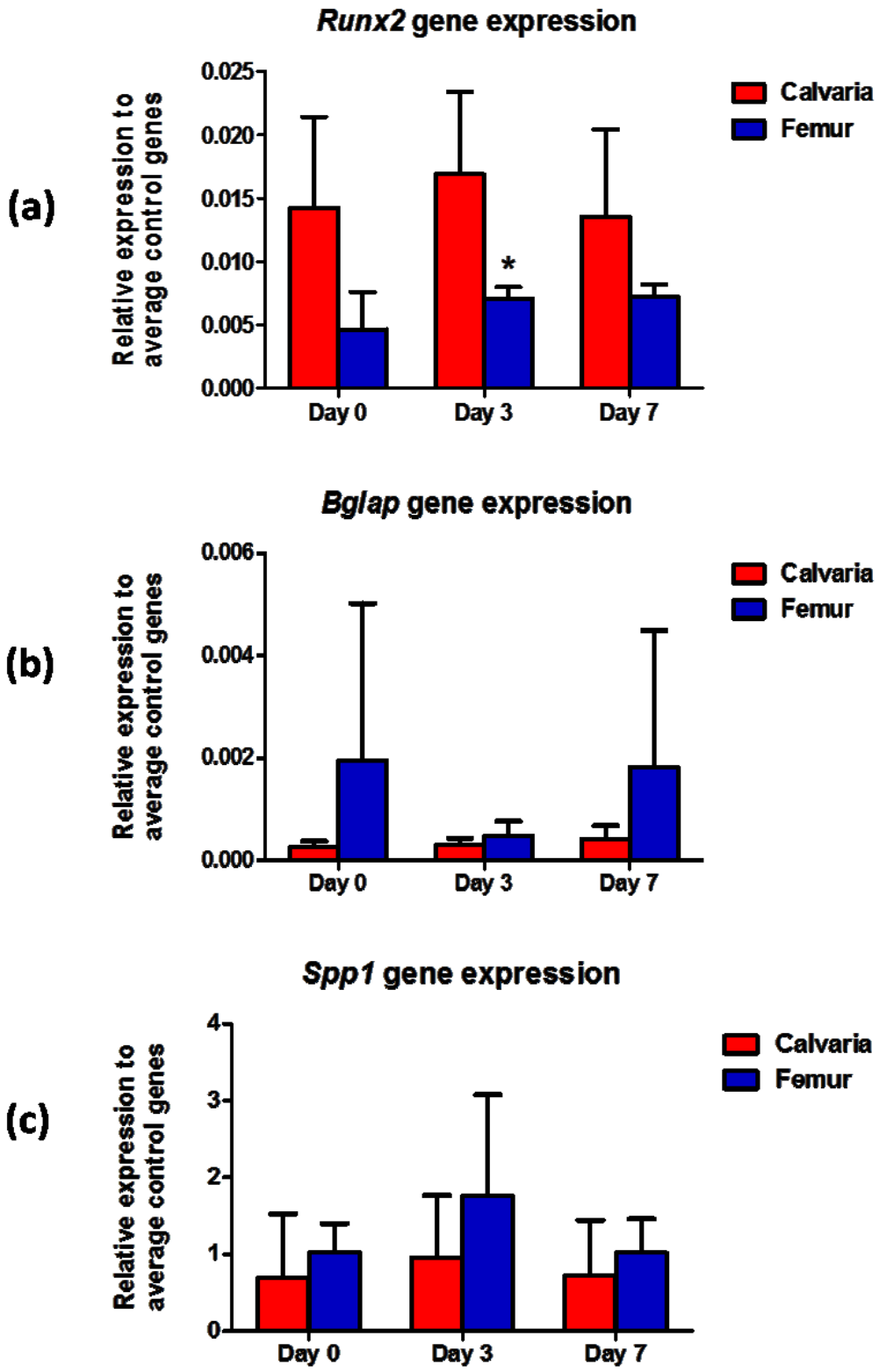


Figure 6-31: Relative expression of *Runx2*, *Bglap* and *Spp1* to average control genes (*Atp5b* and *Eif4a2*). In monotypic culture, calvarial osteoblastic cells stained with CellTracker™ Green, whilst femoral osteoblastic cells were unstained. At Day 0, Day 3 and Day7, the cells passed through cell sorter, grown and collected for RNA extraction, cDNA synthesis and qRT-PCR. Data represented as mean ± SD from 4 different male rats. Data were generated from using 8 different primary osteoblast cultures and 4 replicates were tested for each culture. *Runx2* expressed higher in calvarial osteoblastic cells (a), whilst *Bglap* and *Spp1* were expressed higher in femoral osteoblastic cells (b) and (c). Paired *t*-test was used to compare gene expression between calvarial and femoral osteoblastic cells, *, $p<0.05$, considered significantly different.

b) Femoral osteoblastic cells stained with CellTracker™ Green

In further experiments rat femoral osteoblastic cells were stained with CellTracker™ Green, whilst calvarial osteoblastic cells were unstained. The cells were passed through a cell sorter, grown and collected for RNA extraction, cDNA synthesis and qRT-PCR.

Relative expression of transcription factors (*Msx2*, *Irx5* and *Tbx3*) and osteoblast-associated genes (*Runx2*, *Bglap* and *Spp1*) to average control genes (*Atp5b* and *Eif4a2*) was assessed. Data generated from 4 different male rats expressed as mean ± SD.

Msx2 and *Irx5* were preferentially expressed in calvarial osteoblastic cells (Figure 6-32 a and b), whilst *Tbx3* was preferentially expressed in femoral osteoblastic cells (Figure 6-32 c) from Day 0 to Day 7 in all cultures.

Msx2 and *Irx5* expression appeared to be higher in calvarial osteoblastic cells than in femoral osteoblastic cells, but this was not significantly different (Figure 6-32 a and b). Similarly *Tbx3* expression was not significantly different between femoral and calvarial osteoblastic cells (Figure 6-32 c).

From Day 0 to Day 7, *Runx2* expression was higher in calvarial osteoblastic cells and significantly different at Day 3 ($p<0.05$) (Figure 6-33 a), whilst *Bglap* and *Spp1* expression were not significantly different (Figure 6-33 b and c) from Day 0 to Day 7, except at Day 7 where *Spp1* expression was significantly higher in femoral osteoblastic cells ($p<0.01$).

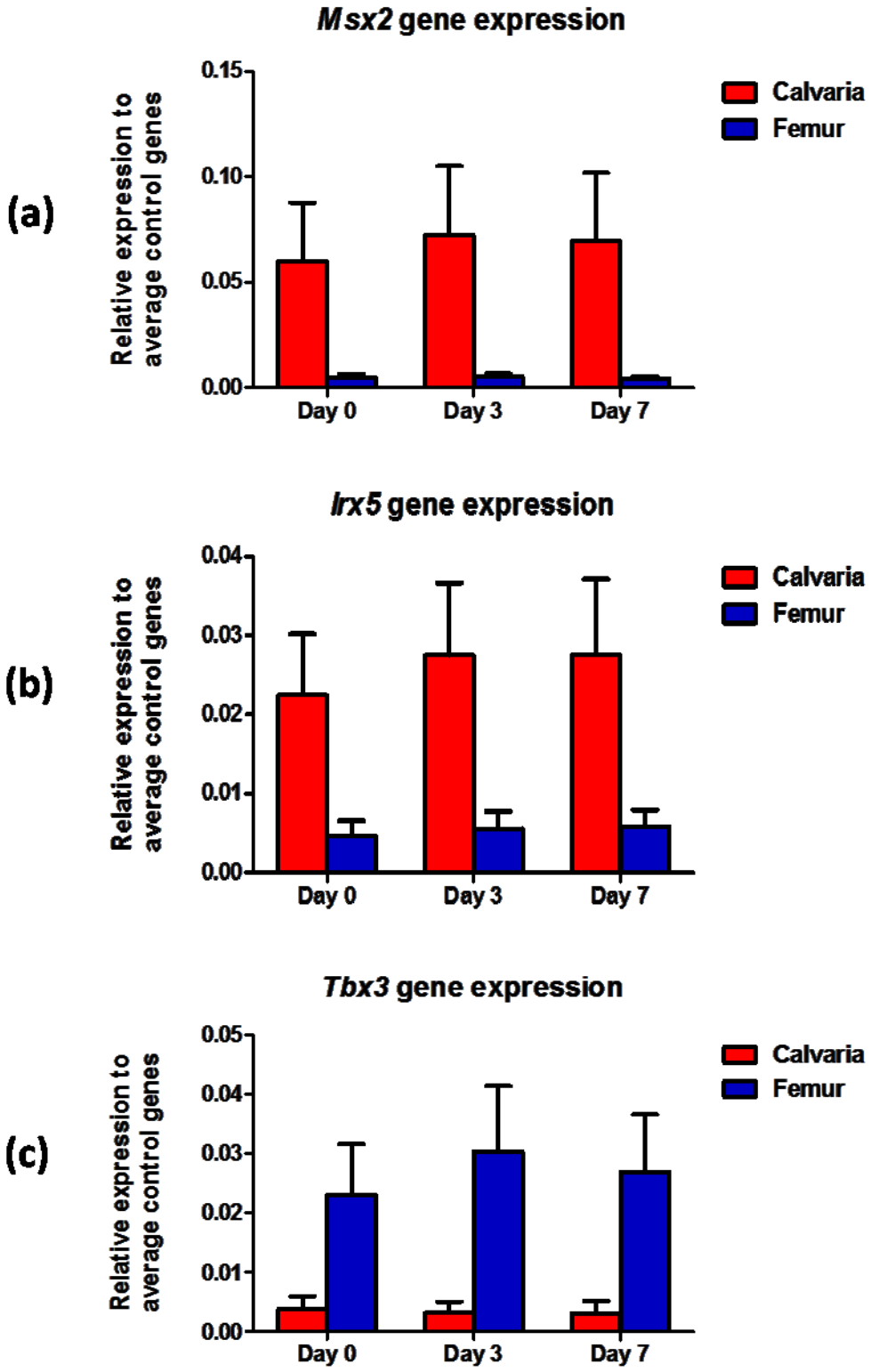


Figure 6-32: Relative expression of *Msx2, Irx5* and *Tbx3* to average control genes (*Atp5b* and *Eif4a2*). In monotypic culture, femoral osteoblastic cells stained with CellTracker™ Green, whilst calvarial osteoblastic cells were unstained. At Day 0, Day 3 and Day7, the cells passed through cell sorter, grown and collected for RNA extraction, cDNA synthesis and qRT-PCR. Data represented as mean ± SD from 4 different male rats. Data were generated from using 8 different primary osteoblast cultures and 4 replicates were tested for each culture. *Msx2* and *Irx5* were preferentially expressed in calvarial osteoblastic cells (a) and (b), whilst *Tbx3* was preferentially expressed in femoral osteoblastic cells (c). Paired *t*-test was used to compare gene expression between calvarial and femoral osteoblastic cells, *, $p<0.05$ considered significantly different.

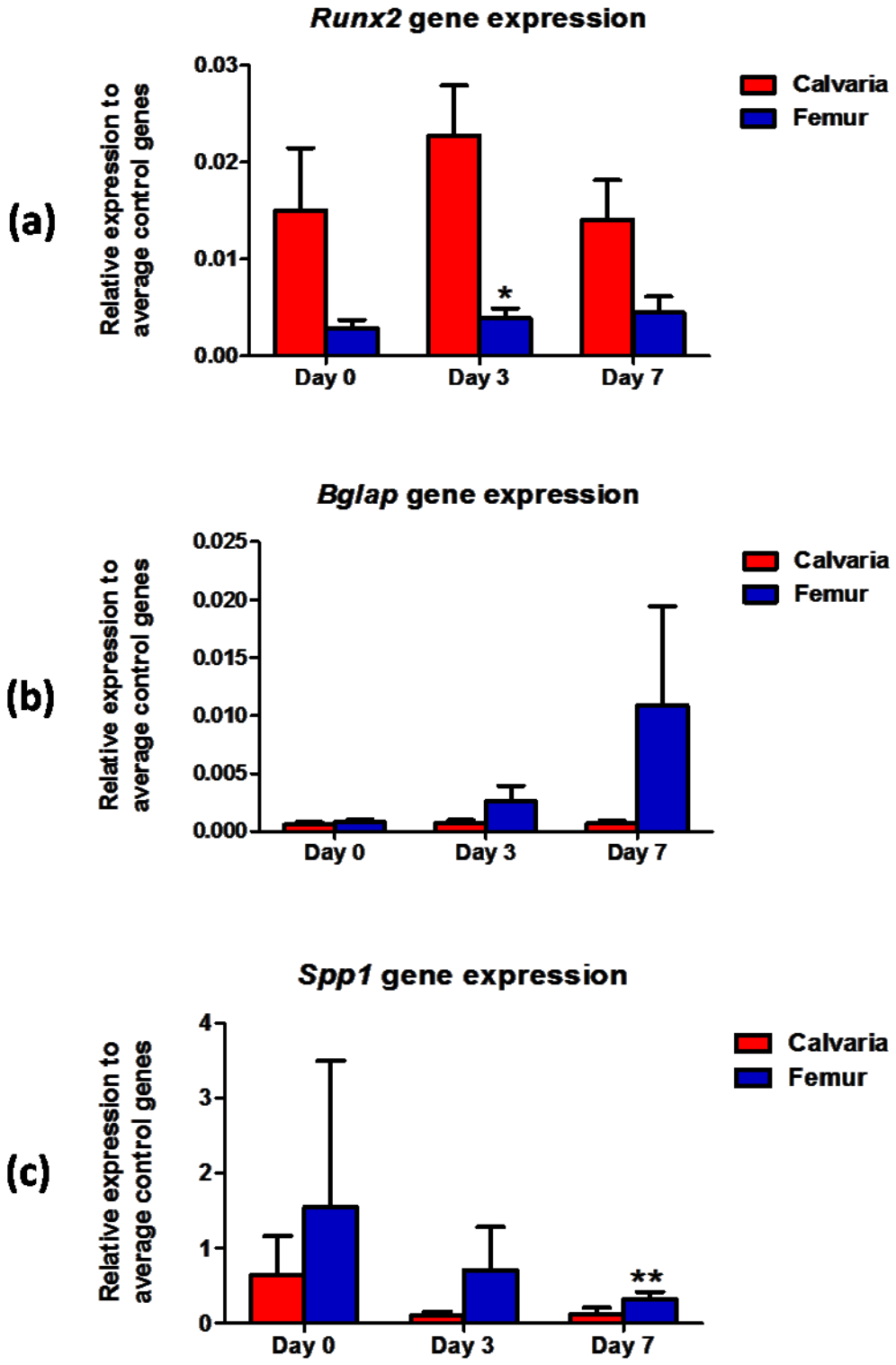


Figure 6-33: Relative expression of *Runx2*, *Bglap* and *Spp1* to average control genes (*Atp5b* and *Eif4a2*). In monotypic culture, femoral osteoblastic cells stained with CellTracker™ Green, whilst calvarial osteoblastic cells were unstained. At Day 0, Day 3 and Day7, the cells passed through cell sorter, grown and collected for RNA extraction, cDNA synthesis and qRT-PCR. Data represented as mean ± SD from 4 different male rats. Data were generated from using 8 different primary osteoblast cultures and 4 replicates were tested for each culture. *Runx2* expressed higher in calvarial osteoblastic cells (a), whilst *Bglap* and *Spp1* were expressed higher in femoral osteoblastic cells (b) and (c). Paired *t*-test was used to compare gene expression between calvarial and femoral osteoblastic cells, *, $p<0.05$, **, $p<0.01$ considered significantly different.

The results for heterotypic cultures using single labelling are described as follows:

a) Calvarial osteoblastic cells stained with CellTracker™ Green

As before, *Msx2* and *Irx5* were preferentially expressed in calvarial osteoblastic cells (Figure 6-34 a and b), whilst *Tbx3* was preferentially expressed in femoral osteoblastic cells (Figure 6-34 c) from Day 0 to Day 7 in all cultures. At Day 0, *Msx2* expression was significantly higher in calvarial osteoblastic cells than in femoral osteoblastic cells ($p<0.05$). *Irx5* was significantly higher expressed in calvarial osteoblastic cells than in femoral osteoblastic cells at Day 0 ($p<0.05$) and at Day 3 ($p<0.01$). At Day 0 and Day 3, *Tbx3* was significantly higher expressed in femoral cells than in calvarial osteoblastic cells ($p<0.01$). There was no evidence of changes in relative gene expression patterns in calvarial cells between day 0 and day 7 of heterotypic cultures.

In osteoblast-associated genes the pattern of gene expression was similar to previously observed, with *Runx2* expression higher in calvarial osteoblastic cells (Figure 6-35 a), and *Bglap* and *Spp1* expression higher in femoral osteoblastic cells (Figure 6-35 b and c). However, there was no significantly difference ($p<0.05$) between calvarial osteoblastic cells and femoral osteoblastic cells in osteoblast-associated gene expression in heterotypic culture. There was no evidence of changes in relative levels of gene expression in calvarial cells between day 0 and day 7 of heterotypic cultures.

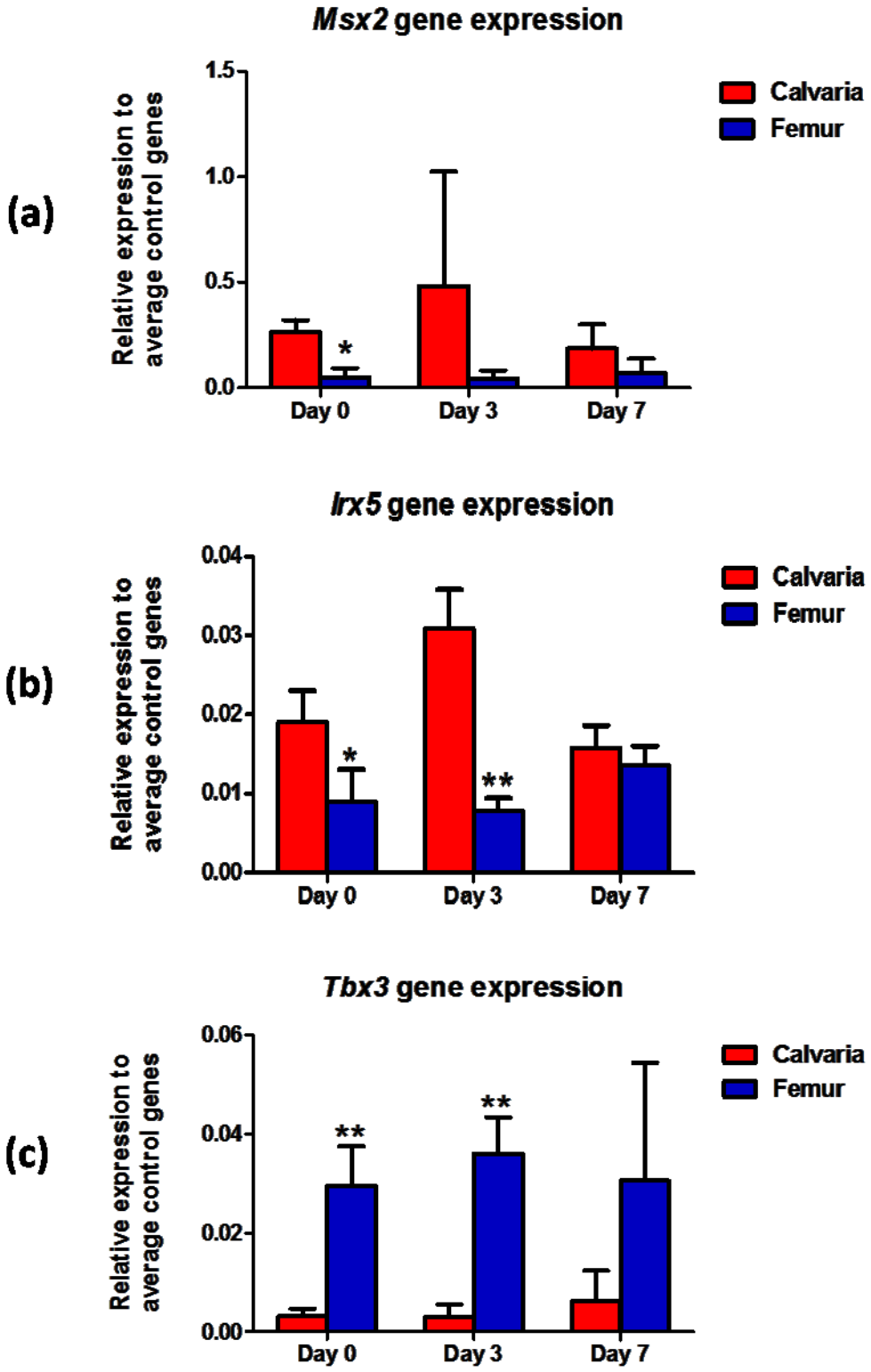


Figure 6-34: Relative expression of *Msx2, Irx5* and *Tbx3* to average control genes (*Atp5b* and *Eif4a2*). In heterotypic culture, calvarial osteoblastic cells stained with CellTracker™ Green, whilst femoral osteoblastic cells were unstained. The cells were grown together in culture for 0, 3 and 7 days. Then they were sorted by cell sorter, continued to grow and collected for RNA extraction, cDNA synthesis and qRT-PCR. Data represented as mean ± SD from 4 different male rats. Data were generated from using 8 different primary osteoblast cultures and 4 replicates were tested for each culture. (a) *Msx2* and (b) *Irx5* were preferentially expressed in calvarial osteoblastic cells, whilst (c) *Tbx3* was preferentially expressed in femoral osteoblastic cells. Paired *t*-test was used to compare gene expression between calvarial and femoral osteoblastic cells (*, $p<0.05$, **, $p<0.01$ considered significantly different).

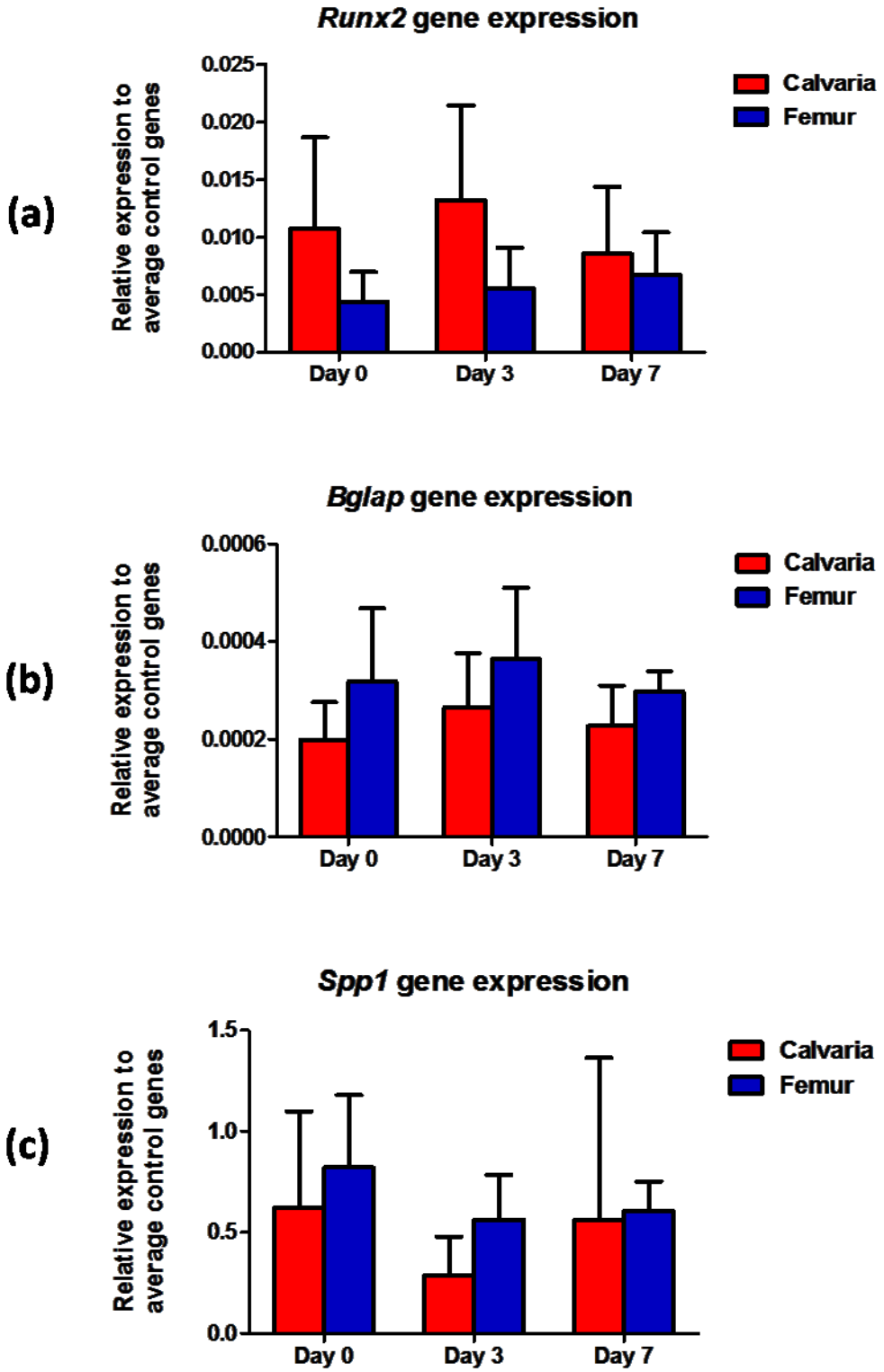


Figure 6-35: Relative expression of *Runx2*, *Bglap* and *Spp1* to average control genes (*Atp5b* and *Eif4a2*). In heterotypic culture, calvarial osteoblastic cells stained with CellTracker™ Green, whilst femoral osteoblastic cells were unstained. The cells were grown together in culture for 0, 3 and 7 days. Then they were sorted by cell sorter, continued to grow and collected for RNA extraction, cDNA synthesis and qRT-PCR. Data represented as mean ± SD from 4 different male rats. Data were generated from using 8 different primary osteoblast cultures and 4 replicates were tested for each culture. (a) *Runx2* expressed higher in calvarial osteoblastic cells, whilst (b) *Bglap* and (c) *Spp1* were expressed higher in femoral osteoblastic cells. Paired *t*-test was used to compare gene expression between calvarial and femoral osteoblastic cells ($p<0.05$ considered significantly different). However, there were no significant differences between the two distinct osteoblast populations.

b) Femoral osteoblastic cells stained with CellTracker™ Green

When femoral cells were labelled, similar results were obtained to those with single staining of calvarial osteoblastic cells. Transcription factors *Msx2* and *Irx5* were preferentially expressed in calvarial osteoblastic cells (Figure 6-36 a and b), whilst *Tbx3* was preferentially expressed in femoral osteoblastic cells (Figure 6-36 c) from Day 0 to Day 7 in all cultures.

At Day 0 and Day 7, *Msx2* expression was significant higher in calvarial osteoblastic cells than in femoral cells ($p<0.01$) and also at Day 0, *Irx5* expression was significantly higher in calvarial than in femoral cells ($p<0.05$). On the other hand, at Day 0 *Tbx3* expression was significantly higher in femoral than in osteoblastic cells ($p<0.05$).

With osteoblast-associated genes *Runx2* expression was higher in calvarial osteoblastic cells (Figure 6-37 a), whilst *Bglap* and *Spp1* expression was higher in femoral osteoblastic cells (Figure 6-37 b and c). *Runx2* expression was significantly different at Day 0 ($p<0.05$), whilst *Spp1* expression was significantly higher in femoral osteoblastic cells at Day 0 ($p<0.05$). As with the calvarial cells, there was no evidence of changes in relative levels of gene expression in femoral cells between day 0 and day 7 of heterotypic cultures.

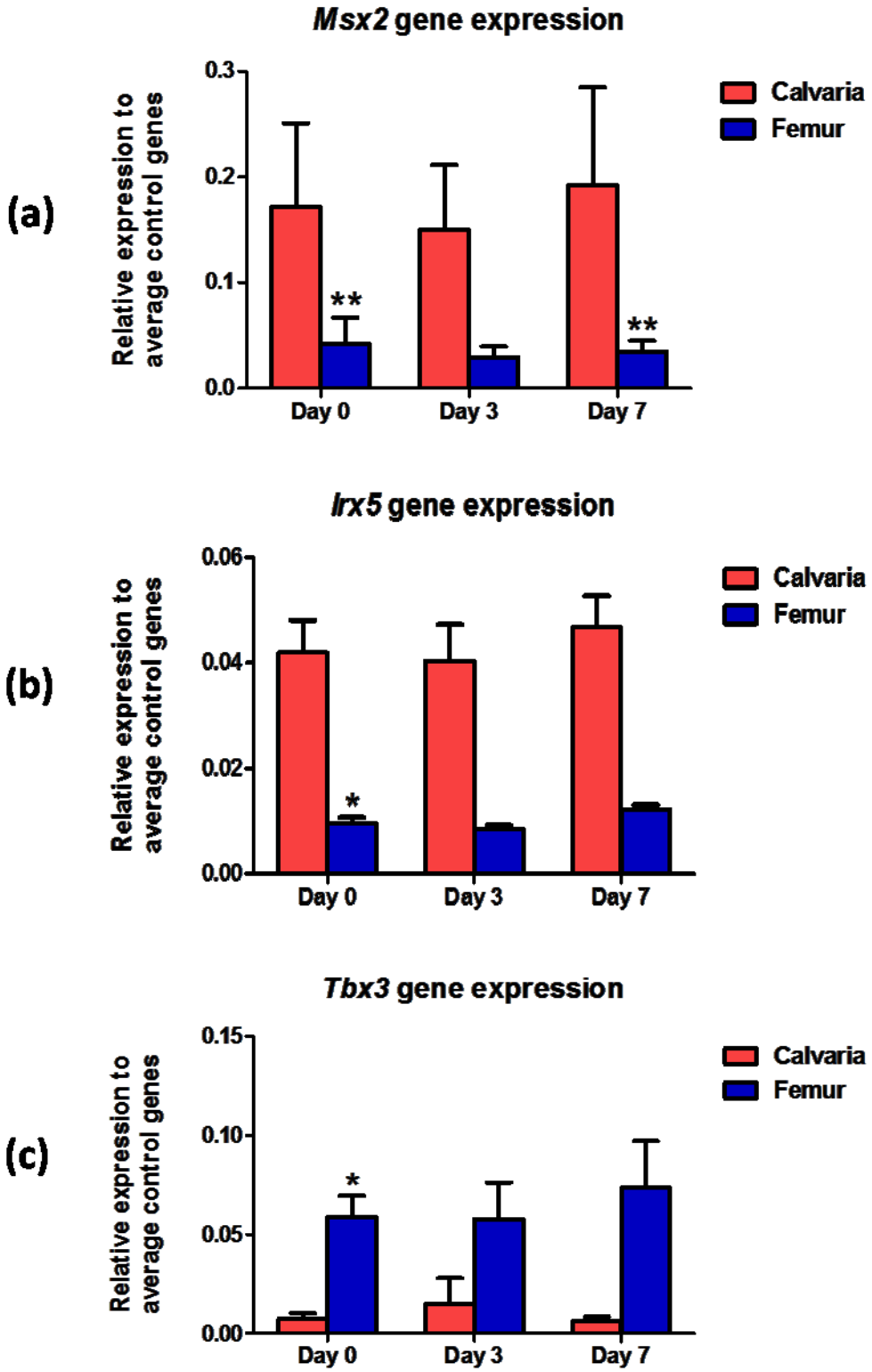


Figure 6-36: Relative expression of *Msx2, Irx5* and *Tbx3* to average control genes (*Atp5b* and *Eif4a2*). *Vice versa*, in this heterotypic culture, femoral osteoblastic cells stained with CellTracker™ Green, whilst calvarial osteoblastic cells were unstained. The cells were grown together in culture for 0, 3 and 7 days. Then they were sorted by cell sorter, continued to grow and collected for RNA extraction, cDNA synthesis and qRT-PCR. Data represented as mean ± SD from 4 different male rats. Data were generated from using 8 different primary osteoblast cultures and 4 replicates were tested for each culture. (a) *Msx2* and (b) *Irx5* were preferentially expressed in calvarial osteoblastic cells, whilst (c) *Tbx3* was preferentially expressed in femoral osteoblastic cells. Paired *t*-test was used to compare gene expression between calvarial and femoral osteoblastic cells (*, $p<0.05$, **, $p<0.01$ considered significantly different).

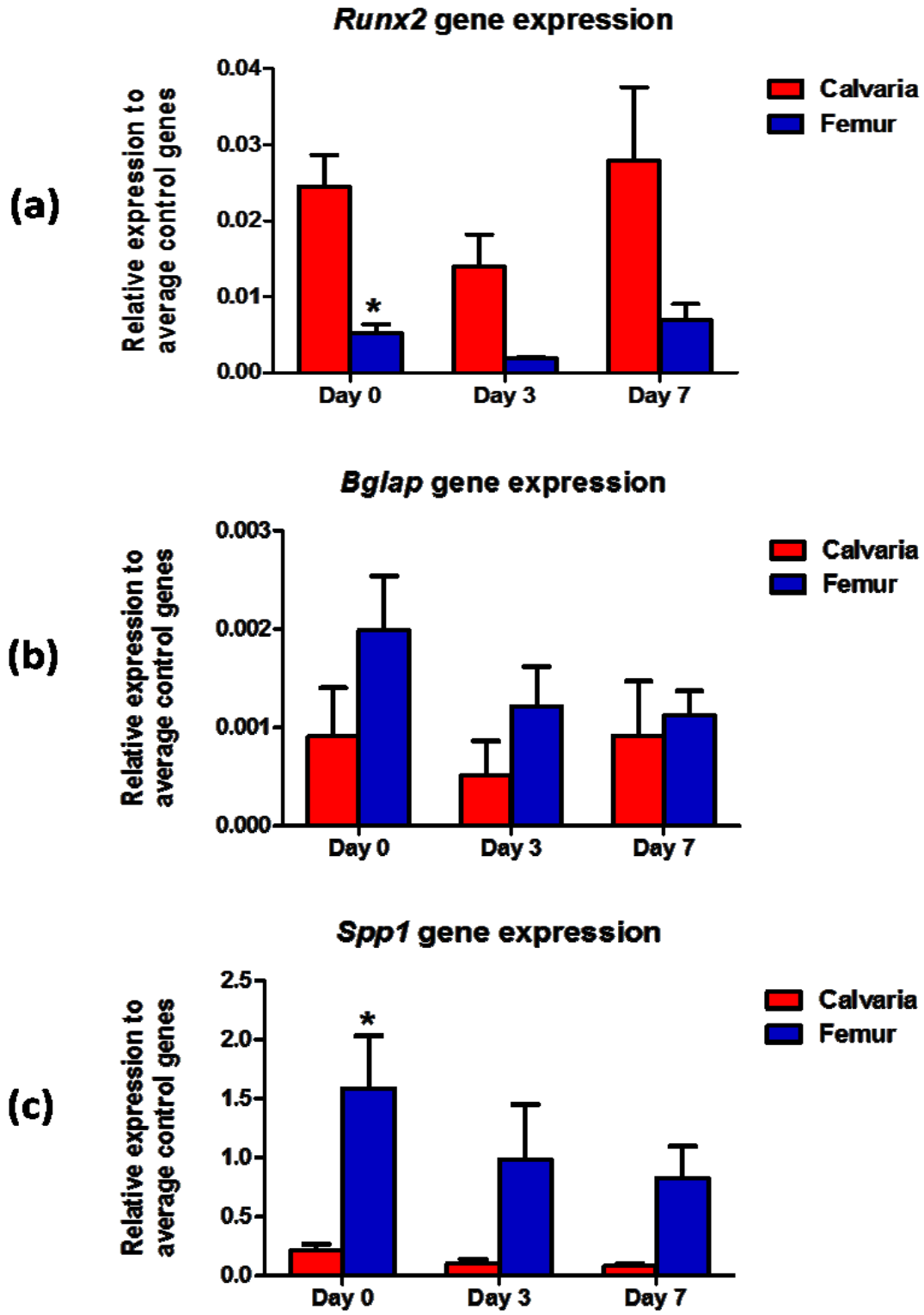


Figure 6-37: Relative expression of *Runx2*, *Bglap* and *Spp1* to average control genes (*Atp5b* and *Eif4a2*). *Vice versa*, in this heterotypic culture, femoral osteoblastic cells stained with CellTracker™ Green, whilst calvarial osteoblastic cells were unstained. The cells were grown together in culture for 0, 3 and 7 days. Then they were sorted by cell sorter, continued to grow and collected for RNA extraction, cDNA synthesis and qRT-PCR. Data represented as mean ± SD from 4 different male rats. Data were generated from using 8 different primary osteoblast cultures and 4 replicates were tested for each culture. (a) *Runx2* expressed higher in calvarial osteoblastic cells, whilst (b) *Bglap* and (c) *Spp1* were expressed higher in femoral osteoblastic cells. Paired *t*-test was used to compare gene expression between calvarial and femoral osteoblastic cells (*, $p<0.05$ considered significantly different).

6.5 Discussion and conclusion

The variation between individuals in gene expression profiles may have an impact on the outcome of these results. Therefore, we reanalysed our data using 8 independent experiments for each matched pair of rat cell cultures. The results confirmed that *Runx2* expression was significantly higher in calvarial osteoblastic cells, whilst *Bglap* and *Spp1* expression was significantly higher in femoral osteoblastic cells at later passages suggesting the difference in their embryonic origin. In addition, *Bglap* was more highly expressed in more mature osteoblastic cells.

Interestingly, our osteopontin production data (Figure 6-7) showed similar production among calvarial osteoblastic cells from 4 rats, whilst showing significant differences among femoral osteoblastic cells from 4 rats. This may be due to variations in *Spp1* gene expression between individual rats or due to osteoblast maturity, with more mature osteoblastic cells showing higher osteopontin production.

Rat 11 showed different expression of osteoblast-associated genes compared to other rats (Figure 6-11 and Figure 6-13). For example, *Runx2* expressed significantly higher in calvarial osteoblastic cells than in femoral osteoblastic cells, except for Rat 11 that there was not different between calvarial and femoral osteoblastic cells (Figure 6-11). In addition, *Spp1* expressed significantly higher in femoral osteoblastic cells than in calvarial osteoblastic cells, except for Rat 11 that there was not different between calvarial and femoral osteoblastic cells (Figure 6-13). This may be due to variation between individual rats or due to difference in osteoblast maturity.

The data support and extend the results of our previous study using microarrays (Rawlinson et al., 2009b) in showing the long term stability and reproducibility of increased osteopontin production in femoral cells. This may be of particular interest in view of the known role that osteopontin may have in mediating the role of mechanical stimulation in bone and might ultimately partly explain why long bones are more susceptible to osteoporosis than skull bones (Rawlinson et al., 2009b; Rawlinson et al., 1995).

Steroid hormones e.g. oestrogens, progesterone and androgens play a crucial role in maintenance of normal bone architecture and cell development (Riggs et al., 2002). By administration of natural or synthetic oestrogen for postmenopausal osteoporosis, the bone loss can be stopped (Benayahu et al., 2000). In this study, we investigated the responses of the regionally distinct cell populations to oestrogen stimulation. The cells were tested and measured by using MTS and EdU incorporation analysed by flow cytometry, especially calvarial osteoblastic cells showed very sensitive or toxic response at 10^{-10} M of oestrogen concentration. This may be due to different batch of commercially perchased oestrogen. Therefore, batch testing is recommended. In addition, pharmacological dose needed to be optimised for each primary cell cultures.

ROS cells (Cheng et al., 1999; Jessop et al., 2001; Migliaccio et al., 1992) and Saos 2 cell lines (Nasu et al., 2000; Rao et al., 2001; Rao et al., 2003) were used as positive controls. However we were unable to demonstrate any consistent effects of oestrogen stimulation on either of the cell populations, or indeed in the positive control cultures (ROS and Saos 2 lines). The reason for this total lack of oestrogen responsive in our cells is surprising given the number of studies that have previously shown that oestrogen can regulate osteoblast functions. Further studies including expression of oestrogen receptors to look at specific protein production such as osteopontin production would be more sensitive and valuable.

Previous studies have demonstrated differences in oestrogen response between skull bones and long bones. ER-α levels were elevated during matrix maturation and then declined during mineralization, whilst ER-β expression was relatively constant throughout differentiation and exhibited more constitutive expression (Wiren et al., 2011). In our studies, we were hoping to extend these observations by testing for differences in responsiveness between the isolated cell populations. Furthermore, it has been reported that there is no difference between female and male rats in the distribution and expression levels of ER-β mRNA in bone (Onoe et al., 1997). However, it might be valuable to investigate these questions again in the future with cells derived from females as well as males.

Overall, our results demonstrate a number of phenotypic and functional differences between femoral and calvarial osteoblastic cells which persisted despite extensive

culture *in vitro*. These include differences in *Spp1, Runx2,* and *Bglap* expression and osteopontin protein production. Despite increased *Bglap* expression in femoral cells, we did not see any differences in osteocalcin production (Chapter 3).

Femoral cells showed markedly increased production of mineralised bone nodules which clearly demonstrated the difference between two distinct osteoblast populations qualitatively. In addition, the size and shape of mineralised nodules appeard to be different between individual rats. Therefore, the mineralised nodules were not quantified by direct counting of nodules. We did not detect any consistent differences in proliferation and ALP expression between the cell populations. These differences match our results from Chapter 4 where the persistence of differences in *Hoxa, Msx2, Tbx3* and *Irx5* expression.

In Chapter 5 we investigated whether *Hoxa* gene expression, as a marker of positional identity and embryological origin and might be reprogrammed by heterotypic cell co-cultures. Previous studies have shown that flow cytometry for sorting purposes provides a simple, reproducible approach to help delineate the stages of bone cell differentiation such as calvarial cells and BMSCs which have been studied for their cell vitality, OPN mRNA expression, alkaline phosphatase activity and cell proliferation, limiting dilution analysis, colony assays, self-renewal capacity, electron microscopy and immunostaining after cell sorting (Zohar et al., 1997). In our study, there were no differences in proliferation and gene expression between the two distinct regional populations in monotypic culture before and after passing through the cell sorter, similar to previous reported (Zohar et al., 1997).

Although we did not demonstrate reprogramming of *Hoxa* expression in our studies, it might have a phenotype switching in heterotypic cultures such as changes in relative expression of populations of *Spp1, Bglap,* and *Runx2*. Rather as expected given the negative results described in Chapter 5, there was no evidence of any changes in phenotypic and functional differences between populations following heterotypic cell cultures.

In conclusion, there was no difference in proliferation and total ALP activity in rat calvarial and femoral osteoblastic cells grown in monotypic or heterotypic cultures. *Spp1* expression was highest among all tested genes and showed the trend of greater expression in femoral than calvarial osteoblastic cells. Our results demonstrated the presence and persistence of potentially important phenotypic differences between the cell populations as seen in early primary osteoblast cultures with greater *Runx2* expression in calvarial osteoblastic cells, whereas *Bglap,* as a late osteoblastic marker, showed no difference between two distinct populations. Moreover, in late primary osteoblast cultures *Runx2* expression was maintained, but expression levels were not different between cell types, whilst *Bglap* expression was significantly higher in femoral osteoblastic cells which were not altered in heterotypic culture experiments.

Chapter 7

Chapter 7: Final discussion and conclusion

7.1 Final discussion

The studies described in this thesis have used novel approaches to investigate whether embryonic positional identity can be maintained, modulated to increase the osteogenic ability and improve regenerative capacity. The findings presented in this thesis have aimed to provide an insight into bone regenerative capacity in tissue engineering by understanding the positioning identity of the cells from different embryonic origin and their modulation may gain more understanding and improve the strategy for bone grafting and some bone-related disease treatment such as osteoporosis.

7.1.1 Cell isolation

Several methods have been used to isolate cells from foetal or adult rats (Declercq et al., 2004) or juvenile mice (Xu et al., 2007). Cell isolation methods may have an effect in the results may be due to the fact that cell isolated from bony tissues were heterogeneous. Some methods can potentially facilitate more homogeneity. In addition, different animals, age of the animal, pooled or matched pairs, different animal species, different in animal sexes may have an effect on result outcome.

Many studies have been done using cell lines e.g. Rat Osteosarcoma (Gu et al., 2001; Maliakal et al., 1994; Noda et al., 1988; Oldberg et al., 1986; Slootweg et al., 1997; Wheeler et al., 2008), MC3T3-E1 (Tobimatsu et al., 2006), Human Osteosarcoma cell line MG-63 (Bonewald et al., 1992; Lajeunesse, 1994; Lajeunesse et al., 1991) and Saos-2 (Hunt et al., 1996). Rat models are well-established for varieties of experiments *in vitro* (Kartsogiannis and Ng, 2004). The studies were carried out primarily using rodents as the findings from these mostly *in vitro* studies could potentially lead to future investigations where there genetics can be manipulated to test findings *in vivo*.

7.1.2 Cell characterisation

Martin and co-workers found that FGF-2 supplementation promoted BMSC proliferation and maintained their multi-lineage differentiation potential during expansion (Martin et al., 1997).

FGF-2 induced GSK-3beta phosphorylation and increased the nuclear levels of beta-catenin in osteoblasts, suggested that FGF may enhance activation of Wnt signalling (Quarto et al., 2010). FGF-2 stimulation of osteoblast differentiation is partially through modulation of the Wnt/β-catenin pathway in *Fgf-2*$^{-/-}$ mice (Fei et al., 2011). In contrast, the previous study by Kalajzic et al., 2003 has shown that FGF-2 and noggin inhibited ALP in primary murine osteoblast differentiation (Kalajzic et al., 2003). In FGF-2 supplemented cultures, the average size of colonies is increased, but colony number is reduced by approximately 30%, when BMSC clones are isolated and expanded in the presence of FGF-2, the frequency of pluripotent clones is increased, the capability of FGF-2 to sustain long-term BMSC cultures maintaining the capacity to differentiate (Muraglia et al., 2000). The sequential supplementation of these cytokines into BMSCs culture might be suitable for cultured bone to use in bone tissue engineering (Maegawa et al., 2007). FGF signalling during posterior body axis extension in mouse and chick embryos have shown that in the extreme posterior FGF signalling is high and maintain the stem zone at the posterior end of the axis in an undifferentiated state (Dorey and Amaya, 2010) that may have an effect on *Hoxa7* gene expression. Based on our data, FGF-2 did not have an effect on *Hoxa* gene expression. However, before using these cells for some other experiments, a wash out period from FGF-2 to avoid the carried-over effect may need to take to consideration. The using of combination of methods to characterise primary osteoblast cultures such as ALP activity, ALP staining, osteoblast-associated markers, bone specific protein production and mineralised bone nodule formation are reccommended.

7.1.3 Persistence of positional identity and transcription factors *in vitro*

Fibroblasts are a mixed population of cells derived from mesenchymal origin and found in numerous tissues. Similar morphology was evidenced in fibroblasts from different sites, but DNA-microarray studies have demonstrated that fibroblasts in different anatomical regions have their own gene-expression profile and characteristic phenotypes, synthesizing extracellular matrix (ECM) proteins and cytokines in a site-specific manner (Chang et al., 2002). The *Hoxa* gene expression pattern in our rat dermal fibroblast was similar to the study in human fibroblasts (Chang et al., 2002; Rinn et al., 2006; Rinn et al., 2008a; Wong et al., 2007).

BMSCs obtained from different anatomical sites e.g. femur, humerus, tibia, ulna and rib have consistently expressed *Hoxa* genes in all BMSCs primary cultures. The pattern of *Hoxa* gene expression in the bone marrow largely matches the pattern of expression seen in osteoblast cultures isolated from these bones (Rawlinson et al., 2009b). Significant differences in the pattern of gene expression in BMSCs culture populations were also seen in rib. This may be due to different embryonic origin.

The rat osteoblastic cells from different embryonic origin from 4 different rats matched pairs retained their positional identity *in vitro* up to 10 subcultures which similar to these studies (Kasperk et al., 1995; Rawlinson et al., 2009b). In a study characterised human bone samples obtained from mandible and iliac crest from four healthy patients showed that phenotypically different human bone cell populations at different skeletal sites (Kasperk et al., 1995).

Crucially, markers of "positional identity" in osteoblasts persisted after many cell doublings in culture despite removing the cells from any environmental cues from their local anatomy. *Hoxa13* was expressed in primary fibroblast cultures derived from dermal tissue overlying the abdominal region, whilst *Hoxa13* was always expressed in primary osteoblast cultures derived from ulnae and femurs. In fact, *Hoxa13* should be expressed in the more posterior region. This may suggest that positional identity is more rigid for skeletal body patterning, whilst it is less fixed in retaining positional information in skin body patterning. Nevertheless, *Hoxa13* maintained the *Hox* pattern of embryo until adult and followed colinearity.

Interestingly, early osteoblast markers such as *Runx2* showed higher expression in calvarial osteoblastic cells, whereas later osteogenic markers such as *Bglap* or osteoblast-associated genes such as *Spp1* were more highly expressed in femoral cells. Thus, from our study neural crest-derived cells may proliferate more, but mesoderm-derived cells may differentiate faster. This is in contrast to Leucht and co-workers who found that neural crest-derived cells showed an increase in expression of osteogenic markers including *Runx2*, *Col1*, *Spp1* and *Bglap* than in mesoderm-derived cells (Leucht et al., 2008a).

In the studies described here, cells from one of the animals appeared to give different results from the other animals. The large variation in expression levels among inbred rats was perhaps surprising but may be due to epigenetic factors. In general, true individual variation provides an explanation why there is such a variable healing response to regenerative procedures.

7.1.4 Can *Hoxa* gene expression and phenotype be modulated by heterotypic culture?

There are a number of different studies previously reported that demonstrate modulation or switching of *Hoxa* gene expression in a range of circumstances. For example, as previously discussed, the studies described by Leucht et al. (2008) demonstrate switching on of *Hoxa* gene expression in *Hoxa*-ve neural crest derived cells following transplantation or co-culture (Leucht et al., 2008a).

Studies of heterotopic transplantations of rhombomere (r) within the rhombencephalic area of chick and quail embryos were investigated and the expression of *Hoxb-4*, *Hoxb-1*, *Hoxa-3*, *Hoxb-3*, *Hoxa-4* and *Hoxd-4* was assessed after a rhombomere was transplanted from a caudal to a more rostral position which expressed the same set of Hox genes as *in situ* (Grapin-Botton et al., 1995). In contrast, their Hox gene expression pattern is modified and expressed genes which activated at the new location of the explants if rhombomeres are transplanted from rostral to caudal and occured whether transplantation was carried out before or after rhombomere boundary formation (Grapin-Botton et al., 1995). The fate of the cells of rhombomeres when caudally transplanted was modified; the rhombencephalic nuclei in the graft extend due to the new location (Grapin-Botton et al., 1995). Transplantations to more caudal regions (posterior to somite 3) showed the induction of *Hoxb-4* in the whole transplant in some cases suggested that neither the mesoderm lateral to the graft nor the notochord is responsible for the induction; the inductive signal emanates from the neural tube itself (Grapin-Botton et al., 1995).

However in our current studies we were unable to demonstrate evidence of switching of *Hoxa* gene expression in heterotypic cultures. This may be more the result of failing to provide the correct environmental cues for Hox switching, than the absence of the potential for switching of *Hoxa* expression in itself.

7.1.5 Future work

In the field of tissue engineering research, enormous work has been done in the past decade to improve three key components including cells, scaffolds and signalling molecules, and particularly to enhance cell capacity for regenerative potential.

In the context of this thesis, we have successfully established rat adult osteoblastic cells matched pairs from two distinct embryonic origin populations. We can grow them over serial subcultures *in vitro* with addition of FGF-2 and importantly they can retain their positional identity and osteoblastic phenotype. These will allow us to further study in depth and try to understand their mechanisms.

7.1.5.1 Experiments using KO-*Hoxa* genes or transfected *Hoxa* genes

If the time allowed, studies of *Hoxa*-KO animal, GFP-transfected *Hoxa* genes, cell signalling, effect on *Spp1* gene expression and osteopontin production would have allowed us to gain further insight about the cell modulation both at cellular and molecular levels. The bones harvested from different sites are different as previously stated. They may also have different bone regenerative capacity. The results of this study contribute to our understanding of why current treatments in bone grafting may be limited in their success.

7.1.5.2 Repeating latest *Hoxa* genes modulation experiments

Surprisingly, in our pilot study, *Hoxa* genes in calvarial osteoblastic cells could be switched in heterotypic culture. However, we were concerned about a false modulation. Therefore, we have thoroughly developed our osteoblast heterotypic model and confirmed our results by repeating with more rat matched paired samples. However, it is still worth repeating these experiments.

7.1.5.3 Experiments testing for cells responsiveness to mechanical loading

Interestingly, *Spp1* gene was more highly expressed of all genes tested and particularly was higher in femoral than calvarial cells. In support of this, at the protein level, osteopontin can be detected by ELISA and also showed that osteopontin production was

significantly higher in femoral than in calvarial cells. It is of considerable interest that calvaria tend to maintain high bone mineral density (BMD) despite not being subjected to mechanical loading and are resistant to the effects of osteoporosis, whilst long bones such as femurs are dependent on mechanical loading for maintenance of BMD and are susceptible to osteoporosis (Rawlinson et al., 2009a). Our data suggest that it would be valuable to study the difference between these two distinct populations in response to mechanical loading using a Bioreactor.

7.1.5.4 Experiments testing for cells responsiveness to oestrogen stimulation

The cells did not show any response to oestrogen stimulation. This might be because these cells were derived from adult male rats. The results could have been different if tested with foetal or young female rats. Other reasons why we could not measure the oestrogen responsiveness could be that the measuring time is a snap-shot and MTS was not sensitive enough to measure the difference. Other possible explanations may be due to the complexity of the system, such as a cascade of gene expression, a networking of hormones or other mechanisms involved that lead to different susceptibility seen at clinical level for example different susceptibility of skull bones and long bones in osteoporosis. Certainly studies of ER receptor expression for specific protein would be more sensitive and useful.

7.1.6 Clinical implications

Although the findings presented in this study are at the 'bench' stage, they could potentially have significant implications for improving bone regenerative capacity for bone regeneration, bone grafting or tissue engineering in the clinic.

The role of fibroblasts in tissue engineering for skin reconstruction has been developed since the 1970s, but little is known about the actual efficacy. Tissue engineering in skin has provided benefits to patients with burns, accidents, infections and chronic wounds. ‘‘Skingineering’’ has various potentials that has just begun to be recognised (Boettcher-Haberzeth et al., 2010). Skin from different anatomical regions have shown different in positional identity (Rinn et al., 2006), and data from our study also suggested that dermal fibroblasts taken from different sites are difference. They can maintain their

embryonic body patterning through adult. This may have an effect on wound healing capacity and when skin grafting procedure performed.

The role of BMSCs in tissue engineering to reconstruct defective bone by composing mixtures of graft materials and autogenous marrow with a variety of different vehicles, is a widely used procedure in orthopedics and oral surgery. Our data suggest that BMSCs and bone from different regions are not the same. This information may need to take into consideration in developing further clinical bone regeneration strategies.

7.1.7 Conclusion

The idea that specific cell phenotypes are similar throughout the body is considered to reflect our normal level of understanding, but our evidence here suggests that this is not the case. The adult primary calvarial osteoblastic cells did not express *Hoxa* genes like femoral osteoblastic cells did. This might be due to the diffence in embryonic origin of osteoblasts derived from paraxial mesoderm and osteoblasts derived from lateral plate mesoderm that seem to have memory to maintain their "*HOX* code" for body patterning from the embryo to the adult organism and may have phenotypic and functional differences.

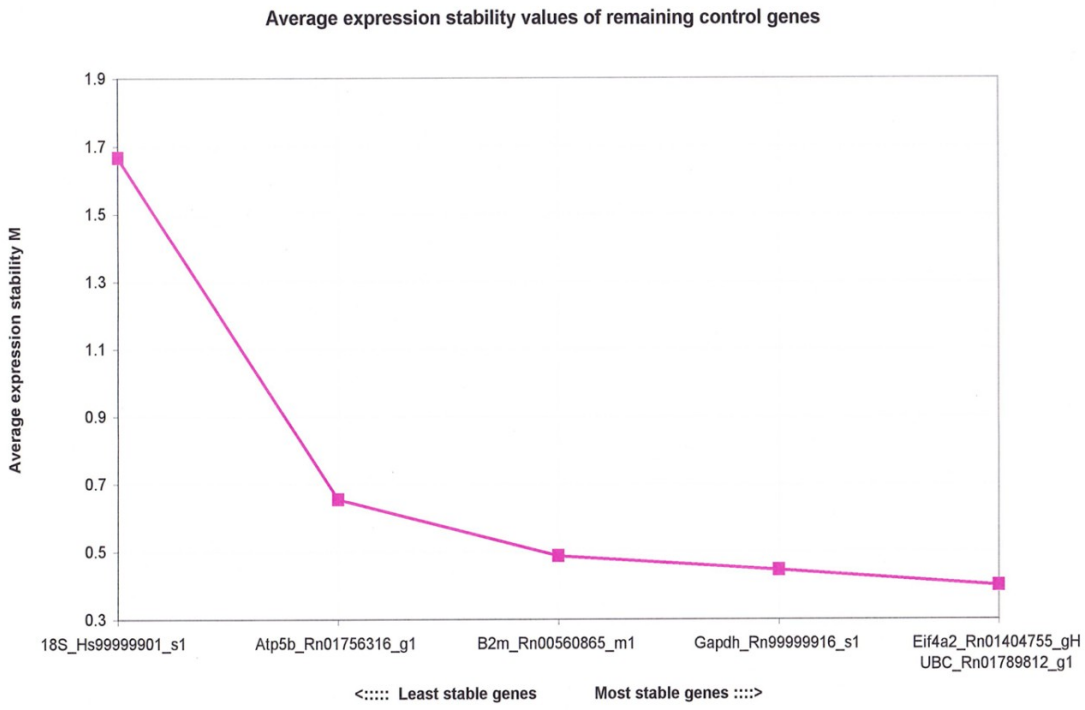
The *Spp1* gene expression data and osteopontin production detected by ELISA suggest that noted phenotypic differences should lead to further studies of the effects of mechanical loading on the distinct cell populations. The ability to enhance cell regenerative potential and alter the path of healing from repair to complete and predictable regeneration will have a significant impact on patients afflicted with unsuccessful bone grafting or regeneration. It is hoped with cell modulation in combination with improvement of scaffold and growth factors, the complete tissue regeneration in tissue engineering triangle can be achieved and by understanding why different bones have different susceptibilities to diseases such as osteoporosis. These could eventually lead to novel therapies being developed.

Appendices

Appendix 1

House Keeping Genes (HKGs) for fibroblasts

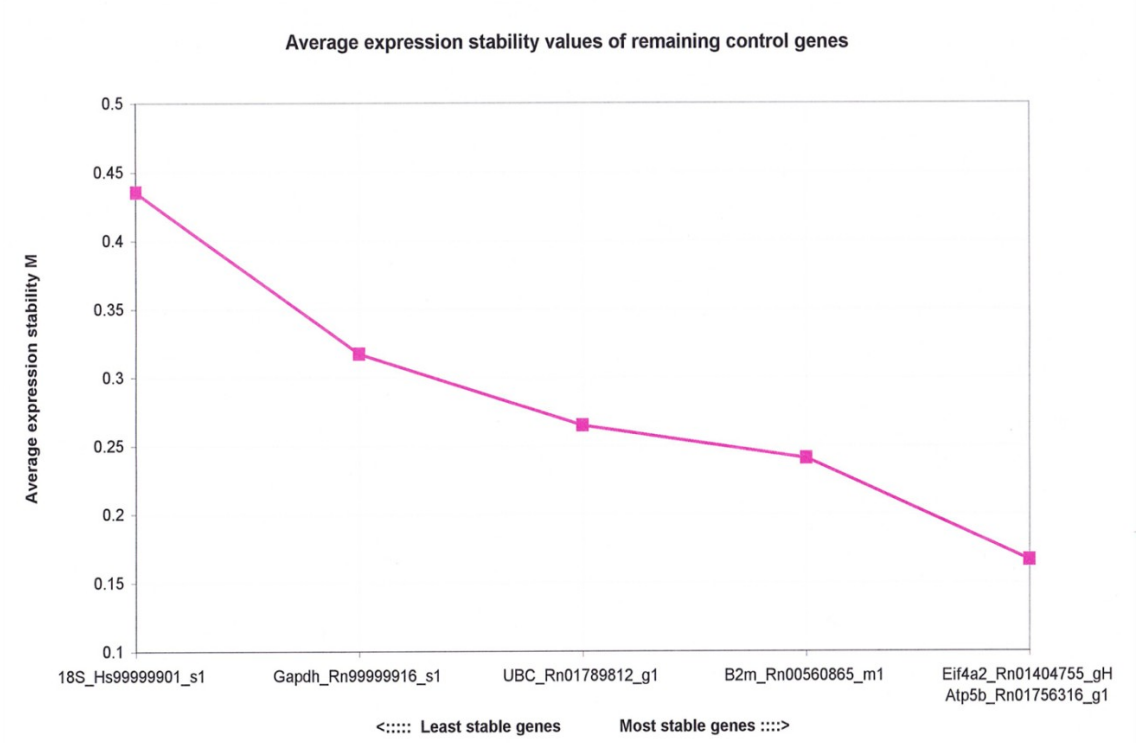
Average expression stability values of six House Keeping Genes tested across all rat dermal-derived fibroblasts used in experiments analysed using geNorm software analysis. The more stable the genes, the lesser the Y axis value. Thus, the most two stable genes, *Eif4a2* and *UBC* were selected as the reference genes for further experiments as show in this figure below.



Appendix 2

House Keeping Genes (HKGs) for BMSCs

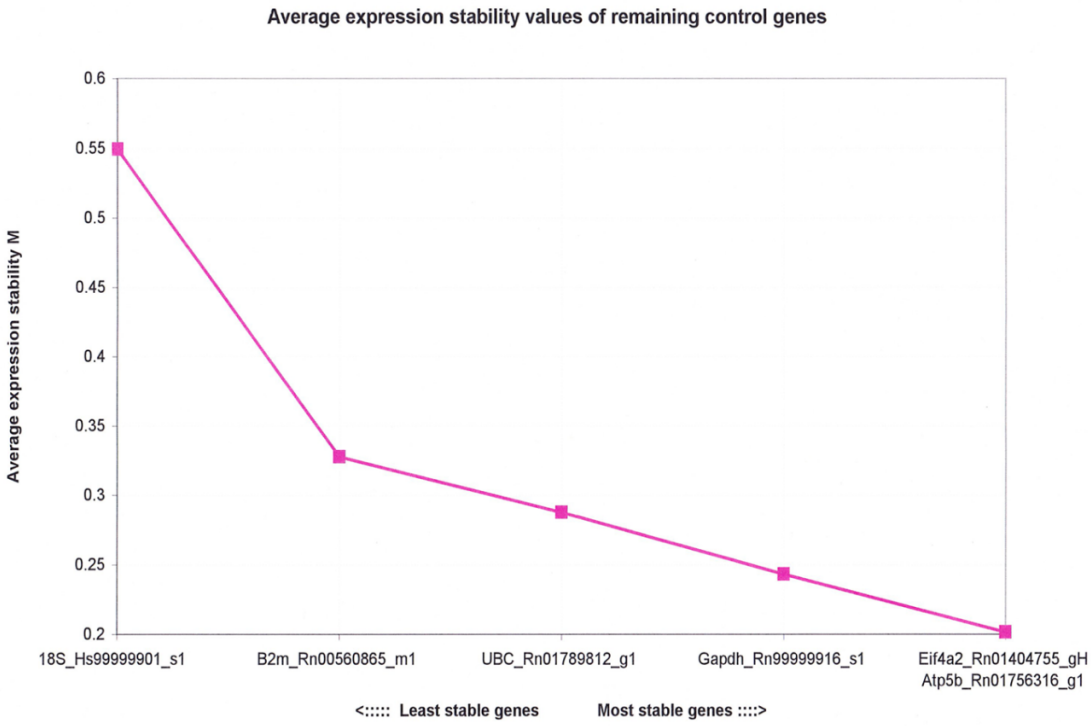
Similar analysis was carried out. Average expression stability values of six House Keeping Genes tested across all rat BMSCs derived from different anatomical sites used in experiments analysed using geNorm software. As previously stated, the more stable the genes, the lesser the Y axis value. Thus, the most two stable genes, *Eif4a2* and *Atp5b* were selected as the reference genes for further experiments as depicted below.



Appendix 3

House Keeping Genes (HKGs) selection for osteoblasts

Similar analysis was carried out and similar results to BMSCs were obtained when tested across osteoblast sample from 4 male rats matched paired. Average expression stability values of six House Keeping Genes tested across 8 primary calvarial and femoral osteoblastic cells used in experiments been tested and analysed using geNorm software. Thus, the most two stable genes, *Eif4a2* and *Atp5b* were selected as the reference genes for further experiments as shown in the figure below.



Appendix 4

Hoxa gene expression in primary dermal fibroblast cultures

1. Data from 1 rat

Hoxa gene expression in primary dermal fibroblast cultures derived from adult rat skin from different regions: scalp, tissue overlying shoulder, abdomen and ulna at passage 5. The data presents the relative expression to average control genes (*Eif4a2* and *UBC*). Negative RT and water were run as negative controls.

At passage 5

	Scalp	Shoulder	Abdomen	Ulna	Negative RT	w a t e r
Eif4a2	0.16965	0.220606	0.187484	0.197004	0	0
UBC	5.958321	4.554302	5.335462	5.099861	0	0
Hoxa1	0	0.002612	0.001499	0.000954	0	0
Hoxa2	0	0	0	0	0	0
Hoxa4	0	0.000821	0.001351	0.000413	0	0
Hoxa5	0	0	0.00187	0.001182	0	0
Hoxa7	0	0	0.005597	0.034982	0	0
Hoxa10	0	0	0.002059	0.001904	0	0
Hoxa13	0	0	0	0	0	0

Note: The Relative Expression (RE) value = 0 was generated from Ct value >35 or undetermined

2. Data from 4 rats

Hoxa gene expression in primary dermal fibroblast cultures derived from adult rat skin from different regions: scalp, tissue overlying abdomen, and tissue overlying femur at passage 5. The data presents the relative expression to average control genes (*Eif4a2* and *UBC*). Negative RT (-ve RT) and water were run as negative controls.

At passage 5

	R9 H	R10 H	R11 H	R12 H	R9 Ab	R10 Ab	R11 Ab	R12 Ab	R9 Fe	R10 Fe	R11 Fe	R12 Fe	-ve RT	water
Eif4a2	2.279	3.004	0.58	0.51	5.68	5.473	0.6522	0.494	2.43	2.15	0.633	0.393	0	0
UBC	0.439	0.333	1.73	1.96	1	0.183	1.5333	2.024	0.41	0.465	1.58	2.547	0	0
Hoxa1	0	7E-05	0	0	0	9E-04	0.0026	5E-04	0	6E-04	0.002	0.006	0	0
Hoxa2	1E-04	6E-05	0	0	0	0	0	0	0	0	0	0	0	0
Hoxa4	0	7E-05	0	0	0	0.003	0.0037	3E-04	0	0.002	0.002	0.003	0	0
Hoxa5	0	5E-05	0	0	0	0.006	0.0083	5E-04	0	0.011	0.014	0.003	0	0
Hoxa7	5E-05	0	0	0	0	0.016	0.0201	4E-04	0.01	0.098	0.066	0.057	0	0
Hoxa10	2E-04	7E-05	0	0	0	0.013	0.0191	9E-04	0.01	0.063	0.059	0.09	0	0
Hoxa13	0	8E-04	0	0	0	0.004	0.0016	2E-04	0	9E-04	0.007	0.034	0	0

Note: The Relative Expression (RE) value = 0 was generated from Ct value >35 or undetermined

Appendix 5

Hoxa gene expression in primary osteoblast cultures

Hoxa gene expression in primary osteoblast cultures derived from adult rat calvarial and femoral bones at passage 5 and 10. The data presents the relative expression to average control genes (*Atp5b* and *Eif4a2*). Negative RT (-ve RT) and water were run as negative controls.

At passage 5

	R9C	R10C	R11C	R12C	R9F	R10F	R11F	R12F	-ve RT	water
Atp5b	0.424115	0.429882	0.419663	0.39274	0.455766	0.448435	1.323273	1.268769	0	0
Eif4a2	2.35785	2.326222	2.382866	2.546214	2.194111	2.229976	0.755702	0.788166	0	0
Hoxa1	0.000144	6.96E-05	0.000228	0.000128	0.002016	0.001181	0.003024	0.001465	0	0
Hoxa2	0	0	0	0	3.98E-05	0	0	0	0	0
Hoxa4	0.000681	0.000165	0.000199	0.000276	0.006968	0.009078	0.015527	0.015554	0	0
Hoxa5	0.000206	0.000228	0	3.13E-05	0.024407	0.025873	0.141258	0.102494	0	0
Hoxa7	4.01E-05	0	0	0	0.097139	0.05746	0.177919	0.127867	0	0
Hoxa10	0	0	0	0	0.007593	0.005179	0.063296	0.062829	0	0
Hoxa13	8.56E-05	0	0	3.64E-05	0.001076	0.002479	0.006993	0.002492	0	0

At passage 10

	R9C	R10C	R11C	R12C	R9F	R10F	R11F	R12F	-ve RT	water
Atp5b	1.690268	1.66069	1.455144	1.332079	1.985965	2.100171	1.619744	1.733135	0	0
Eif4a2	0.591622	0.602159	0.687217	0.750706	0.503534	0.476152	0.617382	0.576989	0	0
Hoxa1	0.000827	0.000372	0.000475	0.000458	0.00334	0.002935	0.005484	0.004215	0	0
Hoxa2	4.95E-05	0	5.03E-05	0	0.000302	0.000176	7.45E-05	0.000243	0	0
Hoxa4	0.000160	0.000293	0.000384	0.000536	0.01826	0.02128	0.011524	0.013573	0	0
Hoxa5	0.000608	0.000164	0.000181	0	0.050538	0.067169	0.035317	0.054598	0	0
Hoxa7	0.000415	0	0.000262	0	0.281505	0.48733	0.257134	0.411652	0	0
Hoxa10	0	0	0.000132	0	0.064842	0.048052	0.023712	0.045634	0	0
Hoxa13	0.000989	0.000694	0.000653	0	0.012395	0.038286	0.058449	0.00026	0	0

Note: The Relative Expression (RE) value = 0 was generated from Ct value >35 or undetermined

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